

Presence and Role of *Acanthamoeba* in Wound Infections

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Abstract

Methicillin-resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa* are the main multi-drug resistant pathogens associated with deep wound infections which then may cause septicaemia. Treatment is problematic and re-infection is quite common. Free Living Amoebae (FLA), such as *Acanthamoeba*, are widely distributed in the environment and may also contaminate wounds. It is well known that *Acanthamoeba* feed on and protect bacteria. The role of *Acanthamoeba* in wound infections is not very well understood. It is possible that the presence of *Acanthamoeba* in wounds is one of the key factors for such re-infections.

In this study, 140 wound swabs were collected to check for the presence of *Acanthamoeba* spp. Only one sample was positive for *Acanthamoeba* spp. Sequencing of the highly variable DF3 region of 18S rRNA gene for the sample showed that this isolate belongs to genotype T4.

In addition, clinical isolates of MRSA and *Pseudomonas* from wound infections were used in this study. The results showed that MRSA and *Pseudomonas* were able to bind with, invade, survive and multiply inside *Acanthamoeba* species. One of the essential compounds for microorganisms to grow is iron. The role of iron chelators, including deferiprone and selected novel compounds based on hydroxyl pyridine moiety, was studied. Findings revealed that all novel iron chelators have an antimicrobial activity against both bacteria. In addition, all novel iron chelators were able to kill *Acanthamoeba*. Cytotoxic effects of MRSA, *P. aeruginosa* and *Acanthamoeba* were investigated using the KB epithelial cell line and mesenchymal stem cells (MSC) using a general caspase inhibitor. The results revealed that the ability of live bacteria to induce cell death was higher compared with heat-killed bacteria, bacteria conditioned medium

(BCM) and *Acanthamoeba* conditioned media (CM). The exact trigger for the cell death in this study was not investigated but the relative contributions of apoptosis and necrosis were investigated using fluorescent technique, caspase inhibition and LDH assay.

In conclusion, presence of *Acanthamoeba* in wounds could be the reason of prolong treatment and reinfection in wounds.

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Abbreviations

(μ, m, k)g	(micro, milli, kilo) gram
(μ, m)M	(micro, milli) Molar
°C	Celsius
18s rRNA	18S ribosomal Ribonucleic acid
ADP	Adenosine diphosphate
AGE	<i>Acanthamoeba</i> granulomatous encephalitis
AIDS	Immune deficiency syndrome
AK	<i>Acanthamoeba</i> keratitis
APS	Ammonium persulfate
ASA	Acanthamoeba-specific amplimer
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BCM	Bacterial conditioned medium
BLAST	Basic alignment search tool
BM	Basement membrane
CA-MRSA	Community-acquired methicillin resistant <i>Staphylococcus aureus</i>
Caspase	Cysteine-aspartic proteases
CFU	Colony forming units
CLED	Cystine lactose electrolyte deficient
CM	Conditioned medium
CNS	Central nervous system
CO ₂	Carbon dioxide
CSF	Cerebrospinal fluid
CT	Cranial computed tomography
DF	Diagnostic fragment
DFO	Deferoxamine
DFP	Deferiprone
DMEM	Dulbecco's Minimal Essential Medium
DMSO	Dimethyl sulphoxids
DNA	Deoxyribonucleic acid
DNA	Deoxyribonucleic acid
dNTPs	2'-deoxynucleoside-5'-triphosphates

Abbreviations

DTH	Delayed-Type-Hypersensitivity
<i>E. coli</i>	<i>Escherichia coli</i>
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EMEM	Eagle's minimum essential medium
ET	Epidermolytic toxin
Exo	Exoenzyme
FACS	Fluorescent activated cell sorting
FBS	Fetal bovine serum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FLA	Free-living amoebae
Fur	Ferric uptake regulator
GAGs	Glycosaminoglycans
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
GTSA	Genotype-specific amplimer
HA-MRSA	Hospital-acquired methicillin resistant <i>Staphylococcus aureus</i>
HAI	Health associated infections
HCl	Hydrochloric acid
HIV	Human immunodeficiency virus
Hla	α -hemolysin
HPA	Health Protection Agency
HPO	3-Hydroxypyridin-4-ones
h	Hour(s)
IFN- γ	Interferon gamma
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
Kb	Kilo base pair
kDa	Kilo dalton
L	Litre
LB	Luria-Bertani
LDH	Lactate dehydrogenase

Abbreviations

LPS	Lipopolysaccharide
MBC	Minimum bactericidal concentration
MBP	Mannose binding protein
MGE	Mobile genetic element
MIC	Minimum inhibitory concentration
min	Minute(s)
MIP-133	mannose induced protein -133
MIP-2	macrophage inflammatory protein-2
MRI	Magnetic resonance imaging
MRSA	Methicillin resistant <i>Staphylococcus aureus</i>
MSCs	Mesenchymal stem cells
MSSA	Methicillin sensitive <i>Staphylococcus aureus</i>
NEAA	Non-essential amino acids
NHS	National health service
NIS	Nonribosomal peptide synthetase
NNA	Non-nutrient agar
OD	Optical density
<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
PAR	Protease activated receptor
PAS	Page's amoeba saline
pb	Base pair
PBP	Penicillin-binding protein
PBS	Phosphate-buffer saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PFA	Paraformaldehyde
PIA	Polysaccharide intercellular adhesion
pmol	Picomole
PMSF	Phenylmethylsulphonyl fluoride
PVL	Panton-Valentine leukocidin
PYG	Proteose peptone-yeast extract-glucose medium
QS	Quorum sensing system
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI-1640	Roswell Park Memorial Institute-1640
RT	Room temperature
SCC _{mec}	Staphylococcal cassette chromosome <i>mec</i>

Abbreviations

SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	<i>Staphylococcus epidermidis</i>
SEA	Enterotoxins serotype A
SG	Stratum germanitivum
sIgA	Secretory immunoglobulin A
spa	Staphylococcal protein A
spp	Species
SSSS	Staphylococcal scalded skin syndrome
TBDR	TonB-dependent receptor
TEMED	Tetramethylethylenediamine
TGF- α	Transforming growth factor alpha
TGF- β	Transforming growth factor beta
TNF	Tumour necrosis factor
Tox	Exotoxin
tRNA	Transfer ribonucleic acid
TSST	Toxic shock syndrome toxin
U	Unit
UK	United Kingdom
UV	Ultra violet light
v/v	Volume for volume
VEGF	Vascular endothelial growth factor
w/w	Weight for weight

Chapter One

1

Literature Review

Chapter 1 : Literature Review

1.1 Structure of skin

Skin is the largest organ of the human body, where it covers an area approximately 1.8 square meters, and it is equivalent to one-sixth of body weight. Each 6 square centimetres of human skin contains roughly 1500 nerve receptors, 3.6 metres of nerves, 4.6 metres of blood vessels, 100 sebaceous glands, and about 350 sweat (Marieb and Hoehn, 2007). It includes two different tissue structures, the epidermis and the dermis, with thickness from 100 μm and 1-4 mm respectively (Figure 1.1).

Skin provides several physiological functions including regulation of body temperature and reduction of harmful effect of ultra violet light (UV). However, the most important function is to provide a protective barrier against harmful microorganisms, mechanical trauma, thermal burn and physical injury.

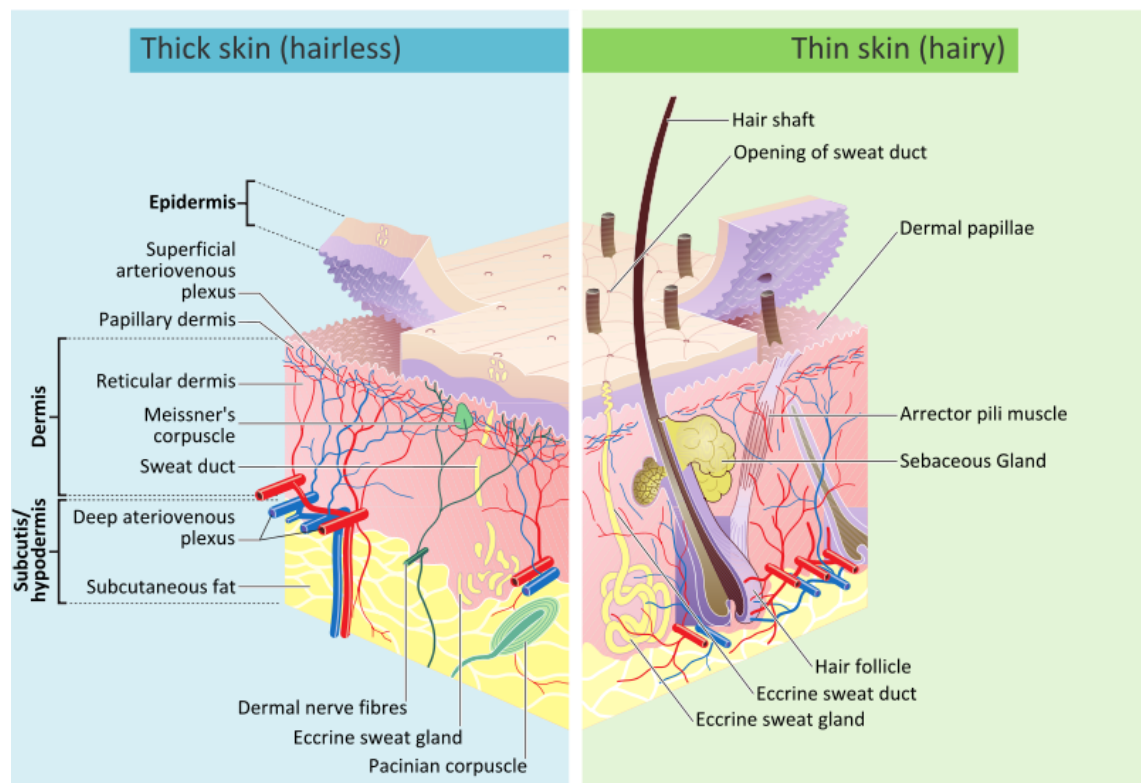


Figure 1.1 Structure of skin.

Non-hairy (right) and hairy regions (left). The epidermal invaginations are called rete ridges. (By Madhero88 and M.Komorniczak - https://en.wikipedia.org/wiki/File:Skin_layers.png, CC BY-SA 3.0, <https://commons.wikimedia.org/w/index.php?curid=21986708> from Wikipedia commons, May 2016).

1.1.1 Structure of the epidermis

The epidermis is part of the first physical barrier of the skin. The thickness of epidermis varies from one part of the body to another. The thinnest epidermis layer is in the eyelids, and the thickest layer is on the palms of the hand and the soles of the feet. Cells in the epidermis layer are constantly renewed from the basal layer below. This part of skin comprises five layers of cells from the basement membrane (BM) to the surface of the skin (Figure 1.2).

1.1.1.1 *Stratum germinativum*

Stratum germinativum (SG) layer is the first layer after BM, and it is the lowest one of the epidermis. It is comprised mainly of the proliferating and non-proliferating keratinocytes, and some of the melanocyte cells. Within this layer, proliferative keratinocytes play role in the continuous regeneration of the epidermis. The new cells will push up the cells in the layers above them which will take about 28-30 days. Although the majority of cells in this layer are keratinocytes, a low number of melanocytes and Merkel cells are present (Borghi and Nelson, 2009; Maricich *et al.*, 2009).

1.1.1.2 *Stratum spinosum*

The Spinosus layer comprises cells which migrate towards the surface of the skin. They are created from the basal cells of the SG layer. Cells in this layer are characterised as polyhedral-shaped containing large nuclei. These cells are able to synthesise proteins, therefore, they are able to produce large amount of the intermediate filament cytokeratin. In addition to the keratinocytes which are the main cells in this layer, it also contains star-shaped immune cells known as Langerhans cells. The function of these cells is to uptake the antigens which have entered the epidermis, then take them to T-

cells of the regional lymph nodes (Romani *et al.*, 2010).

1.1.1.3 *Stratum granulosum*

The keratinocytes moved up from the spinosus layer to create the *stratum granulosum*, which contains between three to five layers of keratinocytes. Cells in this layer are in a late stage of differentiation, and they start to lose their polyhedral morphology, then as a result, they become more flattened. Keratinocytes in this stage start to produce proteins required for cornification, such as K1 keratin filaments. In addition, they begin lose their nuclei, and granulation appears in their cytoplasm (Candi *et al.*, 2005; Palmer *et al.*, 2006).

1.1.1.4 *Stratum lucidum*

This layer is found in the thick skin only, such as on the palms of the hand and the soles of the feet. Cells in this layer do not contain nuclei; and they are flat (Marieb and Hoehn, 2007).

1.1.1.5 *Stratum corneum*

Stratum corneum (SC) cells compose the outer part of the epidermis. This layer consists of 20-30 layers of corneocytes, and forms up to 3/4 of the epithelium thickness (Marieb and Hoehn, 2007). Corneocytes are the most differentiated keratinocytes, which are characterised as polyhedral, nucleated, flattened, dead cells. This type of cell is surrounded by a cornified envelope of cross-linked proteins, and they have an intracellular matrix of hydrophobic non-polar lipids (Candi *et al.*, 2005). The cornified envelope is the principle barrier to inhibit penetration of damaging agents such as chemicals and microbes. It is capable of withstanding mechanical forces and provides the water impermeability of the skin (Madison, 2003).

1.1.2 Structure of the dermis

The dermis is an extensive layer of specialised connective tissue. The main function of this layer is to support the epidermis, however it also provides essential mechanical protection. The dermis is comprised of an extracellular matrix (ECM) that contains glycosaminoglycans, elastins and collagen fibres (Marieb and Hoehn, 2007). The reticular layer is the deepest area of the dermis, which contains nerve endings, sweat glands and oil (sebaceous) glands, hair follicles, and blood vessels (Figure 1.1).

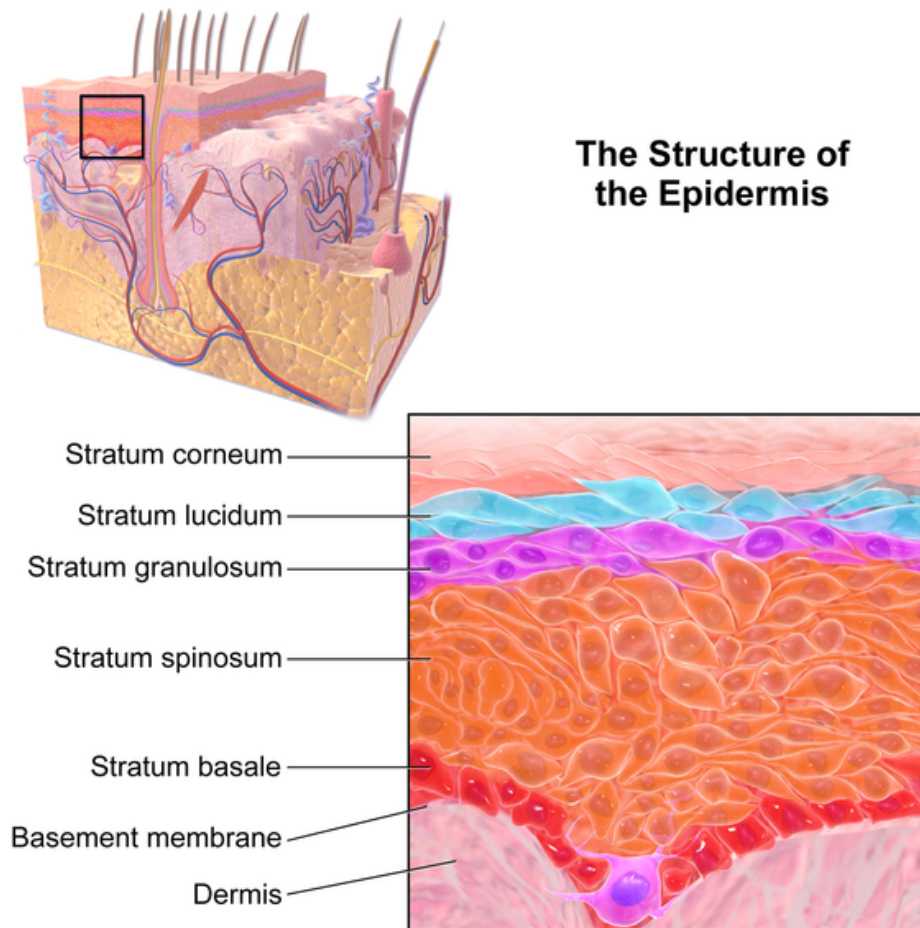


Figure 1.2 Composition of the Epidermis.

The stratum lucidum is a thin translucent layer of dead keratinocytes that is only present in areas of thick skin where it helps to reduce friction and shear forces between the stratum corneum and the stratum granulosum (By BruceBlaus. When using this image in external sources it can be cited as:Blausen.com staff. "Blausen gallery 2014". Wikiversity Journal of Medicine. DOI:10.15347/wjm/2014.010. ISSN 20018762. - Own work, CC BY 3.0, <https://commons.wikimedia.org/w/index.php?curid=30871437> from Wikipedia commons, May 2016).

1.2 Wound and wound healing

The skin provides a natural protective barrier, thus any breach happening in the skin, such as wounds, must be rapidly and expeditiously repaired. To get to this end, skin has the ability to heal itself after any injury or tissue damage. Wound healing is a complex phenomenon which can be described in four distinguishable and overlapping phases (Figure 1.3) (Shechter and Schwartz, 2013).

1.2.1 Haemostasis

This phase starts immediately after the injury of the skin. Wounds cause disruption to the blood vessels which lead to bleeding. As a quick response for the local haemorrhage after endothelial cell injury, the blood platelets get activated and the coagulation process starts. As a result, blood clotting leads to plugging and termination of the bleed.

That would make a temporary barrier to the external environment, and provides a provisional matrix for the invasion of new capillaries within 4 days of the injury (Shechter and Schwartz, 2013). The clot contains platelets and a mesh of fibres derived from the plasma proteins and cleaved by thrombin enzyme (Clark, 2001). Platelets are activated when they aggregate and attach to the collagen surfaces. This activation lets the platelets release chemokines and growth factors such as platelet-derived growth factor (PDGF) and epidermal growth factor (EGF), proteases and vasoactive agents. At the end, fibroblasts get activated by these growth factors to produce collagen. Chemokines have a role in attracting inflammatory cells to the injury area, that leads to the following phase of wound healing (Bauer *et al.*, 1985; Brissett and Hom, 2003).

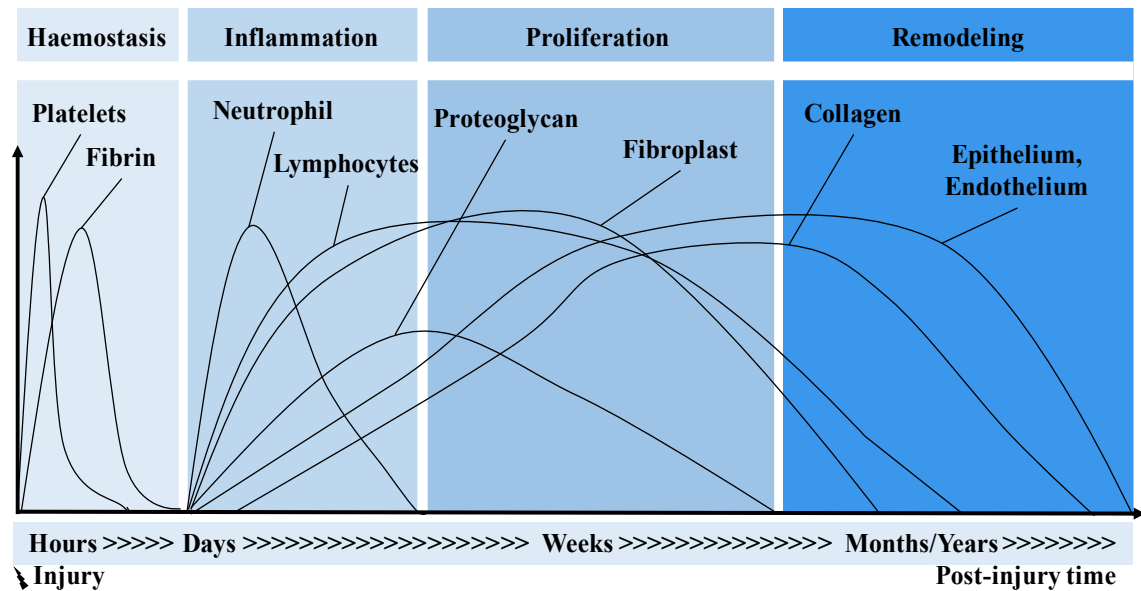


Figure 1.3 Phases of wound healing.

Wound healing is a complex phenomenon which can be described in four distinguishable and overlapping phases: (i) Haemostasis, starts immediately after the injury; (ii) Inflammatory phase, starts within haemostasis up to 3 weeks; (iii) Proliferative phase, starts within 1 week up to 6 weeks; (iv) Remodelling phase, starts after 6 weeks up to 2 years.

1.2.2 Inflammatory phase

The Inflammatory phase begins within the haemostasis phase. In this phase, inflammation is a controlled vascular and cellular response to the injury. The inflammation process is essential to set up the tissue regeneration by clearing the dead cells, foreign materials and germs (Velnar *et al.*, 2009). The response of this phase is characterised by raising the vasopermeability, vessel dilation, and leukocyte permeation (Stadelmann *et al.*, 1998). In addition to the white blood cells, such as granulocytes and monocytes, which infiltrate and are regulated by different types of chemo-attractants, e.g. the cleavage products produced by fibrin and fibrinogen. There are other chemo-attractive agents attracted to a wound site which act as key factors for the inflammatory response, including clotting factors, cytokines, leukotriene, elastin and collagen breakdown products (Velnar *et al.*, 2009).

Granulocytes are a group of leukocytes including neutrophils, which are the first leukocyte cells to arrive at the wound site. They have a protective role in the wound against infections, by killing bacteria via producing toxic reactive oxygen species (ROS). Moreover, they also produce lysosomal enzymes and proteases to debride the injury site. In addition, monocytes attach to blood vessel endothelial cells and infiltrate to ECM. This action protects the wound from contamination via phagocytosis by macrophages (Segre, 2006).

Macrophages are essential cells for wound healing. They release collagenases and elastases, which break down injured tissue, and release cytokines. In addition, macrophages encourage and activate other types of leukocytes to promote the inflammation process. Macrophages have a role in enhancing the apoptosis of irrecoverable cells, and then phagocytosing them. Additionally, they provide many

tissue growth factors, such as transforming growth factor alpha and beta TGF- α , TGF- β , heparin binding epidermal growth factor, fibroblast growth factor (FGF), and collagenase, which activate keratinocytes, fibroblasts and endothelial cells to enhance the healing via regeneration and proliferation of cells (Mosser and Edwards, 2008; Velnar *et al.*, 2009; Guo and Dipietro, 2010).

The last type of leukocytes to enter wounds during the inflammatory phase is lymphocytes. Their number will increase till the end of the proliferative phase and the beginning of remodelling phase (Swift *et al.*, 2001). The lymphocytes are attracted to the injury site by the action of interleukin-1 (IL-1), which also takes part in collagenase regulation. The collagenase is needed later for remodelling collagen, that is a main component of ECM (Velnar *et al.*, 2009).

1.2.3 Proliferative phase

The proliferative phase begins 2-3 days post-injury and takes approximately two weeks. This phase is characterised by the appearance of fibroblasts in the extracellular matrix, which exchange with the provisional network composed of fibrin and fibronectin. Formation of granulation tissue is the main event in this phase, and that can be seen clearly under the microscope. The fibrocytes produce fibroblasts in the connective tissue; however, there is some evidence of the contribution of bone marrow stem cells in producing these fibroblasts (Opalenik and Davidson, 2005). Fibroblasts form an amorphous gel made from glycosaminoglycans (GAGs) and proteoglycans, which play a role in the aggregation of collagen fibres. Fibroblasts migrate and proliferate as a response to different factors, including fibronectin, PDGF, fibroblast growth factor, and TGF- β ; thereafter they are dedicated to the synthesis of extracellular collagen. One-week post-injury, some of the fibroblasts will be transformed into myofibroblasts,

which will connect with each others forming strong contractile tissue to connect wound edges together (Shechter and Schwartz, 2013).

Because of the increase of metabolic demand by the new tissue, the angiogenesis process starts within the proliferation phase, which will be supportive to the provisional matrix. Thereafter, angiogenesis is enhanced by some different growth factors which are produced by macrophages, keratinocytes and vascular endothelial cells such as, basic fibroblast growth factor (FGF-2), vascular endothelial growth factor (VEGF), and TGF- β . Therefore, the flow of blood to the new tissue will increase, thus the healing factors increase as well. These vessels will disappear by apoptosis when the demand for them is reduced (Velnar *et al.*, 2009).

Within hours after the injury, re-epithelialisation will be established by a series of processes such as mobilisation, mitosis, and differentiation of epidermal cells. Keratinocytes in the edge of the wound lose their basal polarity and make pseudopodia from the sides, therefore they interface between clot and healthy dermis to discard the eschar from the viable tissue. When the gap of the wound is closed, the migration of keratinocytes stops and the BM starts to form (Figure 1.4) (Shechter and Schwartz, 2013).

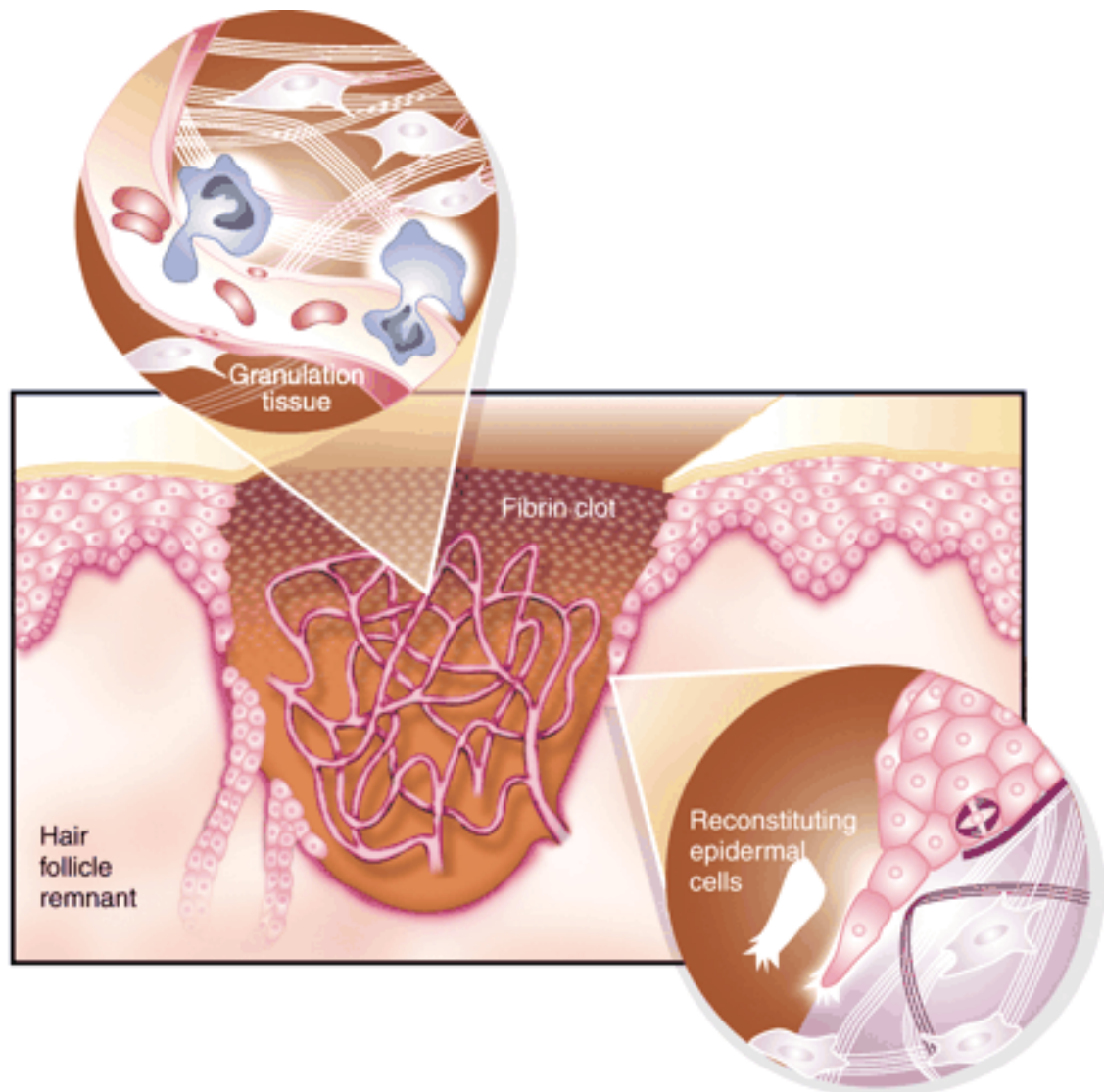


Figure 1.4 The late inflammatory/ early proliferative phase of wound healing.

The granulation tissue is rich in newly sprouted blood vessels, and fibroblasts begin to proliferate and synthesise collagen and ECM components. Keratinocytes of the leading-edge (shown in yellow), begin to re-epithelialise by firstly migrating underneath the eschar to separate it from the underlying regenerating tissue. Figure adapted from (Martin and Parkhurst, 2004).

1.2.4 Remodeling phase

In the remodelling or maturation phase, the collagen fibres are reorganised into a specific structure. Three weeks post-injury, remodelling starts and continues for up to two years. The wound during this phase continues to increase in tensile strength. Collagen type III, which was laid down in the early stage of the wound healing, is replaced by collagen type I until a 4:1 ratio of collagen I to III is reached; similarly the GAGs are decreased until they reach the normal value. High numbers of new blood vessels, myofibroblasts and fibroblasts are degraded through endothelial cell apoptosis. In the end, the newly produced epidermal layer becomes gradually thinner (Velmar *et al.*, 2009).

1.2.5 Chronic wounds (non-healing wounds)

Chronic wounds are those which fail to respond to the wound healing process, resulting in unrepaired wounds. There are many reasons leading to chronic wounds, such as diabetes, pressure, vascular insufficiency, burns, and vasculitis. For example, venous leg ulcers are a common chronic wound that have a high impact on the life quality of the patient and the treatment is not cheap. About 15 to 18 per thousand people in the United Kingdom (UK) have venous leg ulcers, which cost the National Health Service (NHS) between £300 and £600 million *per annum* (Watson *et al.*, 2011; Hodgson *et al.*, 2014). Many factors can disrupt the process of wound healing, which prolong one or more of the wound healing phases, including types of infection, tissue hypoxia, necrosis, exudates, and excess levels of inflammatory cytokines. Prolongation of the inflammatory phase in the wound produces a cascade that conserves a non-healing state. One of the biological markers of chronic wounds is the excessive infiltration of neutrophils to the wound site, and that could be the cause of chronic inflammation. This type of leukocyte produces high amounts of enzymes, notably

collagenase and elastase which break down the ECM and destroy growth and healing factors. It is difficult to treat these wounds unless they respond well to treatment (Maxson *et al.*, 2012). For that reason, researchers are trying to find alternative methods to induce wound healing processes to close wounds. Many studies suggest the possibility of using mesenchymal stem cells (MSCs) in tissue regeneration and wound healing. The presence of MSCs in normal skin and their role in wound healing propose the promise of using exogenous MSCs to treat non-healing wounds (Paquet-Fifield *et al.*, 2009; Zahorec *et al.*, 2015).

1.2.6 Mesenchymal stem cells

MSCs have the potential to differentiate into different tissue-forming cell lineages, e.g. osteoblasts, adipocytes, chondrocytes, tenocytes, and myocytes. Moreover, they can be grown extensively *ex vivo* for several transfers without losing their features and self-renewal capacity (Hocking and Gibran, 2010). MSCs also have the ability to regenerate themselves and control the immune and inflammatory responses. These features give a therapeutic possibility of treating cases with an inflammatory component. In addition, MSCs also have the ability to enhance the surrounding cells' production of biologically active molecules which have a direct effect on cell migration, proliferation, and survival by paracrine signalling. In clinical settings, therapeutic applications of MSCs have shown good results through their role in wound healing phases. Therefore, involvement of MSCs in the wound healing process is very important, especially in these cases of chronic non-healing wounds (Figure 1.5) (Sorrell and Caplan, 2010; O'Loughlin *et al.*, 2013).

Although bone marrow is considered the most common source of MSCs, they are also isolated from different parts of the body, such as adipose tissue, periosteum, tendon,

muscle, synovial membrane, skin, and others. There are some differences in the gene expression and cytokines from different origins, but the expression of the main genes are conserved (Chen *et al.*, 2012), and the features of MSCs are shared between all MSCs from different sources. Moreover, it is still not decided which origin is better for wound healing and other clinical applications. Human placentae become a target for the researcher recently as a source for MSCs. In addition, there are minimal differences of cell phenotype, differentiation and immunomodulative features, between bone marrow-derived MSCs and placental-derived MSCs (Jiang *et al.*, 2012; Mulder *et al.*, 2012).

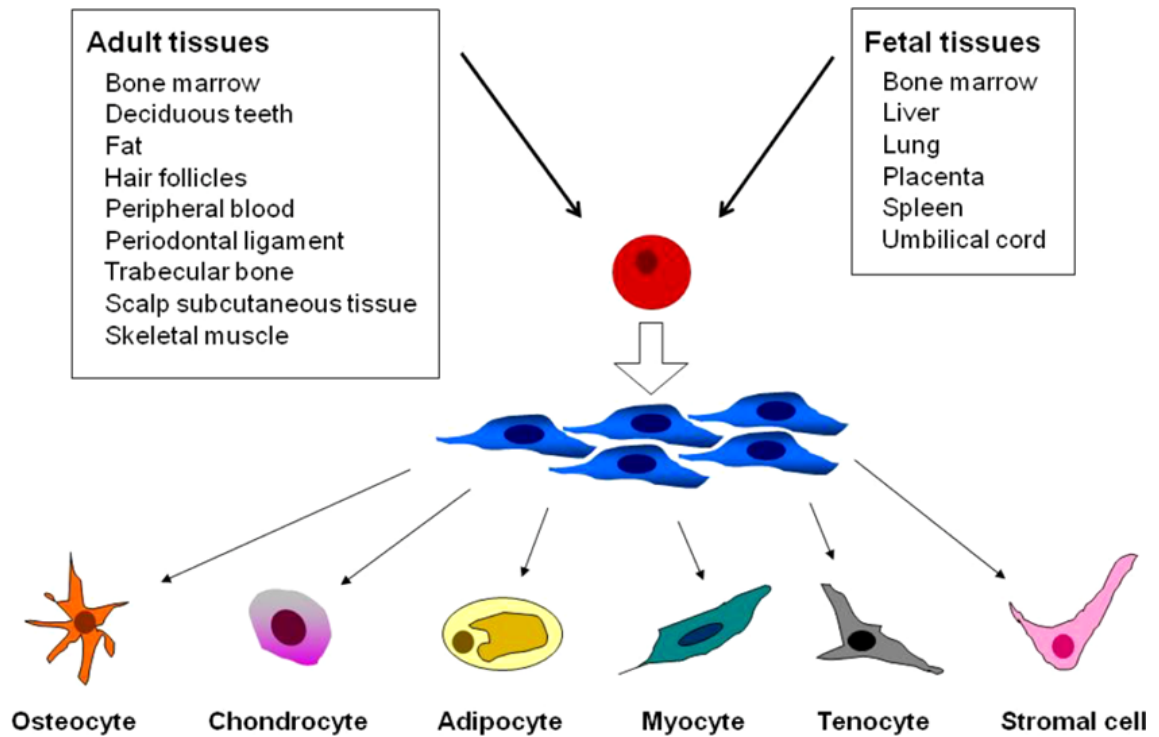


Figure 1.5 Differentiation capacity of MSCs.

Figure adapted from (Claros *et al.*, 2011).

1.2.7 Role of MSC's in wound healing

MSCs' roles are associated with all wound healing phases (Figure 1.6). They show the ability to enhance cells' progress to beyond the inflammatory phase and not develop a chronic state. The presence of MSCs in wounds reduces the production of the proinflammatory cytokines tumour necrosis factor- α (TNF- α) and interferon- γ (IFN- γ), whereas the interleukin-10 (IL-10) and IL-4 will increase (Aggarwal and Pittenger, 2005). Therefore, these features of MSCs give an advantage in treating chronic wounds. In addition, the MSCs have direct and indirect antimicrobial activity. The direct mechanism is through releasing antimicrobial factors (e.g. LL-37), whereas the indirect mechanism is via releasing immunomodulatory factors which have the role of bacterial killing and phagocytosis by the immune cells (Figure 1.6) (Krasnodembskaya *et al.*, 2010; Mei *et al.*, 2010). *In vivo*, MSCs can migrate to the injury site in response to the chemotactic signals of inflammation, then repair the damaged tissue and help in tissue regeneration. Moreover, MSCs improve tissue repair via two mechanisms: differentiation and participation in regeneration of damaged tissue, and paracrine signalling that regulates the cellular response in the injury site (Gnecchi *et al.*, 2008; Chen *et al.*, 2009). The analysis of MSCs' conditioned medium showed their ability to produce many known factors that contribute in tissue repair, such as growth factors, cytokines, and chemokines (Gnecchi *et al.*, 2008). Many cell types e.g. epithelial cells, endothelial cells, keratinocytes, and fibroblasts are responsive to the MSCs paracrine signalling. These responses include cell survival, proliferation, migration, and gene expression (Hocking and Gibran, 2010). In addition, MSCs and MSC-conditioned medium have a role in promoting dermal fibroblasts which speed up wound closure. MSCs enhance dermal fibroblasts to release more collagen type I and alter gene expression (Smith *et al.*, 2010).

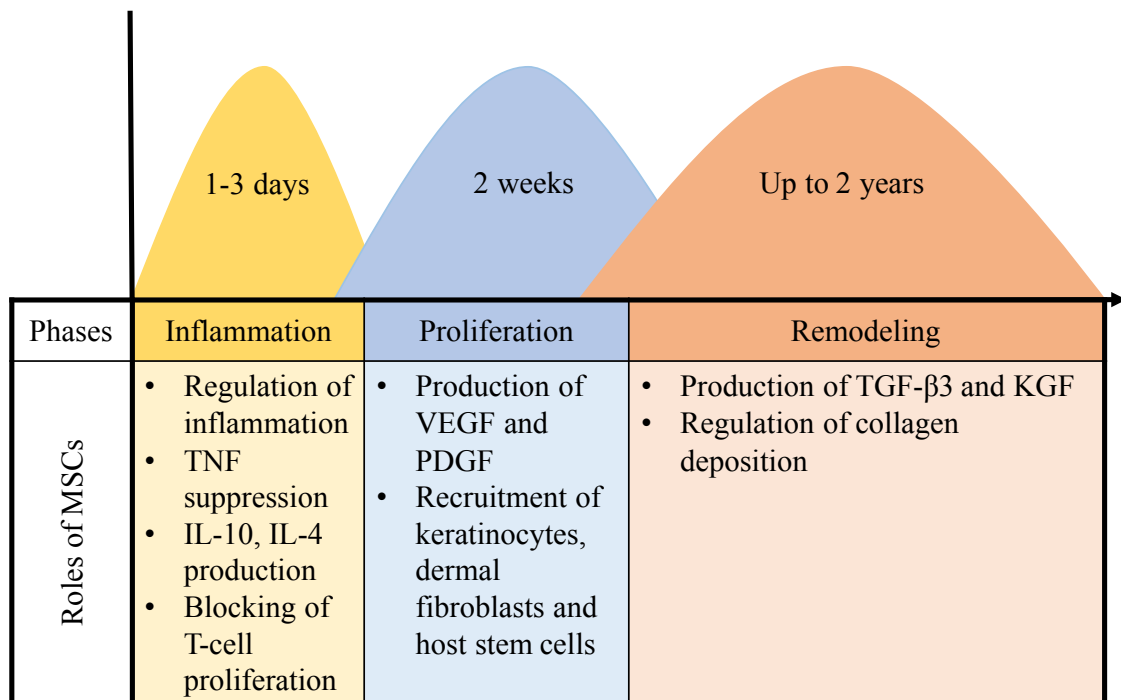


Figure 1.6 Mesenchymal stem cell roles in each phase of the wound-healing process.

1.3 Wound infections

Microbial infection is a serious problem in wounds resulting in increasing the length of treatment, exudate and pain. In chronic infections, it might result in serious consequences such as osteomyelitis, amputation, sepsis and death. In addition, wound infections are the most common cause of morbidity and mortality in patients after operations and also with burns (Melling *et al.*, 2001; Church *et al.*, 2006). Full microbial identification is very important for effective treatment. This is becoming more difficult with the increased number of multi-drug resistant bacteria notably, Methicillin-Resistant *Staphylococcus aureus* (MRSA) (Gray *et al.*, 2005; Soong *et al.*, 2015).

1.3.1 Epidemiology and burden of wounds and wound infections

The accurate global burden of wounds and wound infections is still not completely known. The full picture given by international statistics is difficult to understand, due to regional, national and local specificities for some countries. However, in the developed countries the epidemiology and economic burden of wounds and wound infections is well documented. In North America, there are between 6-7 million patients with wounds each year. About 2.5 million persons in the United States have burns each year. More than 100,000 of these patients are hospitalized, and the mortality is about 12,000 per year (Weinstein and Mayhall, 2003). Another study in the UK showed that 3.55 per 1000 population are patients with wounds. The majority of wounds were surgical 48 %, leg/foot (28 %) and ulcers (21 %) (MacDonald, 2009).

Wound infections could lead to severe circumstances and increased morbidity and mortality among patients. The burden of high cost on health-care is increasing (Zoutman *et al.*, 1998). In the UK, the cost was estimated about £9.89 million between 2006 and 2007, including dressing, full-time nurses and hospital beds. This is a huge amount of money spent on wound infections only. Moreover, the cost of surgical

wounds has proved difficult to estimate (Lynch *et al.*, 1992), however, hospital costs alone could be over £1500 per patient (Zoutman *et al.*, 1998).

A study in the USA among paediatric patients with and without surgical wound infections showed that wound infections prolonged hospital stay by 10.6 days, and that costs patient care about US\$ 27288 per patient (Sparling *et al.*, 2007). Similar study by Bond and Raehl showed that hospitalised patients over 65 years old with wounds were three times more likely to die compared with healthy people (Bond and Raehl, 2007).

1.3.2 Pathogenesis of wound infections

Bacteria (e.g. *Pseudomonas aeruginosa*), fungi (e.g. *Aspergillus*), protist (e.g. *Balamuthia mandrillaris*) or viruses (e.g. human immunodeficiency virus (HIV)) may enter any wound through a break in the skin. This could happen through external sources such as, direct contact (e.g. transfer from hands of a carer or equipment), environment (e.g. microorganism from the surrounding air), and self-contamination (e.g. from the patient's own skin (Sibbald, 2004; Sibbald *et al.*, 2007)). The host immune system will be triggered when the microorganism establishes, proliferates and releases enzymes and toxins that lead to local inflammation. As a result, there will be some signs and symptoms that characterize wound infection, including erythema, oedema, elevated temperature, pain, unusual wound odour and/or purulent exudates. Local infection may become systemic if host defence mechanisms are not enough to eradicate the infection or the therapy used is inappropriate (Nichols, 1985).

The presence of pathogens in wounds does not always lead to wound infections (Thomson and Smith, 1994). Wound healing and infection are affected by the relationship between the virulence of pathogen and the host resistance to control the growth of bacteria in wound (Sibbald *et al.*, 2007). Three important factors are

associated with infection or/and wound healing. They are: infectious dose, pathogen virulence and host resistance (Dow *et al.*, 1999). Therefore, should the microorganisms invade and multiply inside tissues to cause an infection, that can be apparent clinically by the presence of toxins, intracellular replication or antigen-antibody response (Sibbald *et al.*, 2007).

1.3.3 Classification of wounds with bacterial imbalance

Classification of wounds in a *continuum* has been used to provide guidance on the route to administer systemic antibiotics. Schultz *et al.* (2003) described four levels of microbial interaction (Figure 1.7):

1.3.3.1 Colonised

This level has a very low number of microorganisms, and this wound is considered to be progressing normally. The main signs of this level are no necrotic tissue, presence of low number of pathogens on the surfaces, and there may be pink-purple epithelial tissue (Sibbald *et al.*, 2007; Gray *et al.*, 2010).

1.3.3.2 Critically colonised

In this level, the number of microorganisms is increased, and this wound is considered to be not responding to appropriate therapy. The main signs in this level are a wet wound, no new necrotic tissue, wound easily bleed with any touch and the tissue may be unhealthy with a dark colour (Gray *et al.*, 2010).

1.3.3.3 Locally infected

Number of pathogens in this level is high, and wound has some cellulitis on the adjacent skin or around the wound about 2 cm from the wound edge (Dow *et al.*, 1999). In this level, microorganisms are present within a wound and in deeper tissue, there are necrotic tissues in the absence of cellulitis, tissue may be an unhealthy deep red colour,

wound bleeding with any slight touch. Moreover, wound in this level is wetter than wound in critically colonised (Kingsley, 2001).

1.3.3.4 *Spreading infection*

Number of microorganisms in this level is very high. They are found on the surface of wound and in deeper tissue. The wound is surrounded by cellulitis about 2 cm from the edge. The main signs in this level are similar to those in locally infected. Furthermore, odour may be offensive, and there may be pyrexia, lymphangitis or lymphadenitis or bacteraemia (Schultz *et al.*, 2003; Sibbald *et al.*, 2003; Gray *et al.*, 2010).

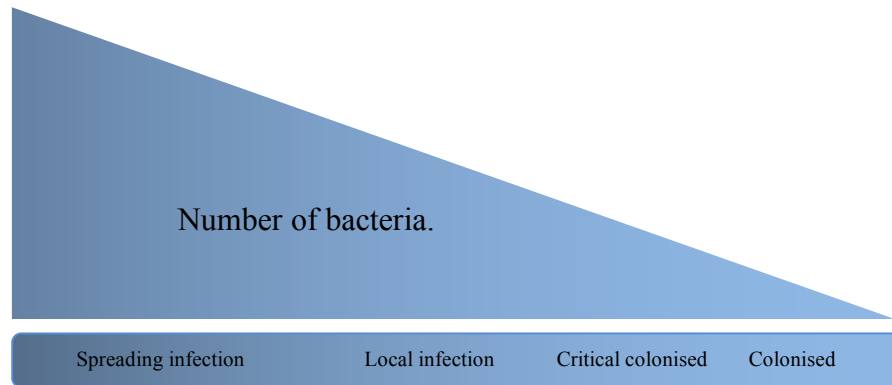


Figure 1.7 Classification of wounds *continuum*.

Four levels of microbial interaction with wounds, starts with colonisation that shows low number of bacteria and no signs or symptoms of infection, then critical colonisation that shows an increasing number of bacteria with no signs and symptoms, next stage is local infection that shows an increasing number of bacteria with ability to invade the tissue and presence of signs and symptoms, next stage is spreading infection that shows a very high number of bacteria and could lead to bacteraemia.

1.3.4 Common wound pathogens

Wounds may be infected by numerous microorganisms including bacteria, fungi, protist and viruses. Bacteria are the most common pathogens that lead to wound infection (Table 1.1). Some bacteria have a positive effect on wound healing, such as the skin commensal *Staphylococcus epidermidis* (SE) found as flora on the skin (Schultz *et al.*, 2003; Rajan, 2012).

Sibbald *et al.* (2003) reported that wounds infected of less than a month duration post-infection seems to have a high percentage of Gram-positive bacteria, while wounds that have been infected for more than a month post-infection tend to have Gram-negative bacteria present followed by anaerobes. Another study showed that delayed wound healing is not dependent on one specific microorganism or group of bacteria, but is caused by the presence of four or more different species of microorganisms (Trengove *et al.*, 1996). In addition, wounds are considered as infected when about 10^5 colony-forming units (CFUs) is present in one gram of viable tissues (Gardner *et al.*, 2001; Percival *et al.*, 2012; McCarty and Percival, 2013).

Most Common Bacteria Causing Wound Infections

Species	Total isolates	Percentage of isolates
<i>Staphylococcus aureus</i>	2602	44.6
<i>Pseudomonas aeruginosa</i>	648	11.1
<i>Enterococcus</i> spp	542	9.3
<i>Escherichia coli</i>	422	7.2
<i>Enterobacter</i> spp	282	4.8
<i>Klebsiella pneumoniae</i>	248	4.2

Table 1.1 The most common bacteria that cause wound infection.

Table adapted from (Rajan, 2012).

Each bacterial strain can have a quite different approach to cause disease or change its sensitivity to antibiotics. Because of the overuse and misuse of antibiotics, new multidrug resistant strains are emerging. Most of these antibiotic-resistant bacteria are hospital-acquired.

Most common antibiotic-resistant pathogens in wound infections are (Serra *et al.*, 2015):

1.3.4.1 Methicillin-resistant *Staphylococcus aureus* (MRSA)

Staphylococcus aureus are Gram-positive cocci. Staphylococci were observed and cultured for the first time by Pasteur and Koch, but Ogston in 1881 and Rosenbach in 1884 made more studies on them (Ogston, 1881). The cocci are usually arranged in grape-like clusters. Staphylococci may be found as part of the normal flora of other specific sites such as the upper respiratory tract, and are commonly present on animals. The major pathogen within the genus is Methicillin-sensitive *Staphylococcus aureus* (MSSA), and they cause a wide range of hospital-acquired and community-acquired infections notably skin and wound infection (Creech *et al.*, 2015).

Methicillin-resistant *Staphylococcus aureus* (MRSA) were described directly after the introduction methicillin in 1961 in the UK. Methicillin was introduced to solve the problem of penicillin-resistant strains that became high during 1950s. The presence of MRSA in many hospitals has become a major public health issue, with concern expressed by patients and members of the public about the clinical implications (Bukharie, 2010).

Resistance to methicillin in *S. aureus* is mediated by PBP-2a, a penicillin binding protein, encoded by the *mecA* gene (Mulligan *et al.*, 1993; Ben-David *et al.*, 2008). The gene which is located on the staphylococcal chromosome cassette (SCCmec), permits the organism to grow and replicate in the presence of methicillin and other β -lactam

antibiotics (Hiramatsu *et al.*, 2001; Peacock and Paterson, 2015). There are two mechanisms responsible for resistance of *S. aureus* to β -lactam antibiotics. The first one is the low affinity of PBP-2a to β -lactam, and the second is the releasing of β -lactamases that damage the antibiotics (Mulligan *et al.*, 1993; Edgeworth, 2011).

1.3.4.1.1 Virulence factors

Staphylococcus aureus strains have a large number of surface-associated and extracellular factors, such as enterotoxins serotype A (SEA), toxic shock syndrome toxin-1 (TSST-1), cytolytic toxins (α and β haemolysins) exfoliative toxins, Pantone-Valentine leukocidin (PVL), protein A, and several enzymes (Table 1.2.) (Vandenesch *et al.*, 2003). Those factors probably play an important role in the ability of the organism to overcome the body's defences and cause infections (Table 1.2.). They bind to tissues and secrete exotoxins, however the entire process is not fully understood (Patel *et al.*, 2012).

Enterotoxins, types A-E, G, H, I and J, are commonly produced by up to 65 % of staphylococcus strains. These toxic proteins are heat stable, withstanding exposure to 100°C for several minutes (Greenwood *et al.*, 1992). The enterotoxins and the TSST-1 cause toxic shock and related illnesses through induction of massive cytokine release, from both macrophages and T lymphocytes (McCormick *et al.*, 2001). The recent community-acquired MRSA (CA-MRSA) isolation rate has shown evidence of increased virulence resulting in increased prevalence of toxic shock cases and more severe soft-tissue infections and in many cases increased mortality. However, TSST-1 can be produced by hospital-acquired MRSA (HA-MRSA) as well as MSSA strains and, therefore, TSST-1 production should not be considered a hallmark of CA-MRSA strains. The absence of circulating antibodies to TSST-1 is a factor in the pathogenesis of this syndrome (Fey *et al.*, 2003; Bukharie, 2010).

Another important virulence factor in *S. aureus* is PVL, a member of the recently described family of synergohymenotropic toxins. PVL damages the membranes of host defence cells through the synergistic activity of two separately secreted but non-associated proteins, LukS-PV and LukF-PV, causing tissue necrosis (Lina *et al.*, 1999). Although some investigators have suggested that PVL expression does not correlate directly with polymorphonuclear leukocyte lysis (Saïd-Salim *et al.*, 2005), PVL producing CA-MRSA isolates has been increasingly associated with necrotizing pneumonia and necrotizing cutaneous infections (Lina *et al.*, 1999; Francis *et al.*, 2005; Bukharie, 2010).

The other virulence factor are epidermolytic toxins (ETs). Two kinds of epidermolytic toxin (ET-A and ET-B) are commonly produced by *S. aureus*, which cause blistering diseases (Greenwood *et al.*, 1992). ET allows *S. aureus* to proliferate and spread beneath the skin. These toxins induce intra-epidermal blisters at the granular cell layer. The most dramatic manifestation of ET is the scalded skin syndrome in young children, where the toxin spreads systemically in individuals who lack neutralizing antitoxin (Ladhani *et al.*, 2001).

Virulence Factors Of *Staphylococcus aureus*

Virulence factor	Activity
Cell wall polymers	
Peptidoglycan	Inhibits inflammatory response; endotoxin-like activity
Teichoic acid	Phage adsorption; reservoir of bound divalent cations
Cell surface proteins	
Protein A	Binds to Fc region of IgG
Clumping factor	Binds to fibrinogen
Fibronectin-binding protein	Binds to fibronectin
Exoproteins	
α -Lysin	Impairment of membrane permeability; cytotoxic effects on phagocytic and tissue cells
β -Lysin	
γ -Lysin	
δ -Lysin	
Panton-Valentine leucocidin	Dermo-necrotic
Epidermolytic toxins	Cause blistering of skin
Toxic shock syndrome toxin	Induces multi-system effects; super antigen effects
Enterotoxins	Induce vomiting and diarrhoea; super antigen effects
Coagulase	Converts fibrinogen to fibrin in plasma
Staphylokinase	Degrades fibrin
Lipase	Degrades lipid
Deoxyribonuclease	Degrades DNA

Table 1.2 Some virulence factors of *S. aureus* and their activities.

Table adapted from (Greenwood *et al.*, 2012).

1.3.4.1.2 Treatment

S. aureus and other staphylococci are inherently sensitive to many antimicrobial agents. Among the most active benzylpenicillin, about 90 % of strains found in hospitals are now resistant. Resistance to penicillin depends on production of the enzyme penicillinase, a β -lactamase that cleaves the β -lactamase ring. MRSA strains are resistant to all β -lactam agents, and often to other agents such as the aminoglycosides and fluoroquinolones. Glycopeptides (vancomycin or teicoplanin) are the agents of choice in the treatment of MRSA infection, but these agents are expensive and may be toxic. Currently, there are four approved antibiotics for the treatment of MRSA infections, including vancomycin, linezolid, daptomycin and tigecycline (Boucher, 2008; Van Hal *et al.*, 2011).

1.3.4.2 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative, aerobic bacillus belonging to the family pseudomonadaceae. Almost all strains are motile by one or two polar flagella. *Pseudomonas aeruginosa* is a free-living bacterium, it could be found in soil and water. It is a typical species of its group that contains 12 other members. Most of the clinical cases caused by *P. aeruginosa* can be associated with the immunocompromise such as patients with burns. Many cases of *P. aeruginosa* infection can be attributed to general immunosuppression (e.g. AIDS patients) (Wu *et al.*, 2011; Percival *et al.*, 2012; McCarty and Percival, 2013).

1.3.4.2.1 Virulence factors

As mentioned earlier, *P. aeruginosa* can be found in different environments, which enables the bacteria to grow and survive in almost any environment. However, *P. aeruginosa* rarely causes community-acquired infections in immunocompetent patients.

Virulence factors of *Pseudomonas* are classified into two main groups, cell-associated and extracellular virulence factors (Castillo-Juárez *et al.*, 2015).

Moreover, up to 50 % of hospitalised patients are at high risk of *P. aeruginosa* colonisation (Van Delden and Iglewski, 1998). An important virulence factor of *P. aeruginosa* that is probably responsible for the association of this bacterium to epithelium is type 4 pili. There are several other non-pilus adhesions responsible for the binding to host epithelial cells which have been described, but their role in infection is still unclear. Another virulence factor is flagella, which are primarily responsible for motility, and it may also have a role in adhesion to epithelial cells (Alborzi *et al.*, 2009).

Most *Pseudomonas* strains produce two exotoxins (exotoxin A and exoenzyme S), and a variety of cytotoxic substances including proteases, phospholipases, rhamnolipids and the blue pigment pyocyanin (De Bentzmann *et al.*, 2000). Exotoxin A is responsible for local tissue damage and bacterial invasion (Vidal *et al.*, 1993). Purified exotoxin A is fatal for mice, and that supports its role as a main systemic virulence factor of *P. aeruginosa*. Exoenzyme S is responsible for direct tissue destruction in lung infection and may be important for bacterial dissemination (Van Delden and Iglewski, 1998). There are other enzymes which are produced by *P. aeruginosa*, such as haemolysins, phospholipase C and rhamnolipid. These factors may work together to break down lipids and lecithin. Moreover, both may contribute to tissue invasion by their cytotoxic effects.

Production of fluorescein *in vivo* allows *P. aeruginosa* to compete with mammalian iron-binding proteins such as transferrin. In association with pyocyanin, it gives rise to the characteristic blue-green pus of pseudomonas-infected wounds and hence the old species name *pyocyanea* (Wu *et al.*, 2011; Greenwood *et al.*, 2012).

1.3.4.2.2 Treatment

Pseudomonas aeruginosa is normally resistant to many antimicrobial agents. Among β -lactam compounds, piperacillin, ticarcillin, ceftazidime and carbapenems are usually active, as are aminoglycosides such as gentamicin and tobramycin. Aminoglycosides are often used in combination with a β -lactam antibiotic. This provides the potential for antibacterial synergy and reduced antibiotic resistance, but there is little evidence of clinical superiority. Monotherapy with broad-spectrum β -lactam agents such as ceftazidime and imipenem is used, but resistance to these agents occurs. Fluoroquinolones such as ciprofloxacin exhibit good activity against *P. aeruginosa* and penetrate well into most tissues, but resistance may develop. Polymyxins are usually active, but they are toxic and generally reserved for topical application (Estahbanati *et al.*, 2002; Kucisec-Tepes *et al.*, 2006; Kucisec-Tepes and Antolić, 2014).

Passive vaccination may be useful for the treatment of septicaemia and burn infections due to *P. aeruginosa*, which are associated with a high mortality rate. Active vaccination to prevent pulmonary colonisation would be desirable for patients with cystic fibrosis and several conjugate vaccines are in clinical trial (Estahbanati *et al.*, 2002).

1.3.5 Cell death

Cell death can be natural as a normal process of the cell cycle developing an organ's tissue and preventing sickness or functional disorder. However, cell death can also be induced by pathogens such as bacteria, viruses (DeLeo, 2004; Shao *et al.*, 2004), fungi (Glass and Dementhon, 2006) and protist (Lee *et al.*, 2002; Raina and Kaur, 2012). Host cell death is a defence response to the host-microbe interaction process. Specific chemical signals in a particular stage of infection are expressed to activate specific

molecules that are responsible, leading to cell death. The virulence factors produced by bacteria have the main role in host cell damage. Past and recent studies have demonstrated two different types of cell death; these are apoptosis and necrosis. Those two cell death types are widely connected with bacterial infection. Therefore, the trying of understanding the pathways of cell death that induced by bacterial infection have been shown a significant attention for the researchers (Hossain, 2012)

1.3.5.1 *Characteristics of apoptosis and necrosis*

Both apoptosis and necrosis are forms of cell death, however, there are significant differences between them. Apoptosis is considered as a programmed cell death which is required for the growth and the survival of multicellular organisms. Apoptosis is triggered by two different pathways, receptor-mediated (extrinsic pathway) and mitochondria-mediated (intrinsic pathway). However, necrosis occurs by ROS production or danger signals, such as lysosomal destabilization, releasing non-lysosomal cysteine proteases (calpain), and reduction of Adenosine triphosphate (ATP), that are induced by bacterial infection or physical damage. In comparison with the necrosis, apoptosis does not enhance inflammatory responses which are induced with the necrosis process, with the removal process of discrete cells (Rathmell and Thompson, 2002). Furthermore, the apoptotic bodies of apoptotic cells exist as vesicles containing the cellular content, such as mitochondria, lysosomes and Golgi bodies. In contrast, the necrotic cell' membrane is lysed and ruptured, therefore all the cell contents are released to cause inflammatory responses. Moreover, apoptosis is an active process of individual cell death, whereas necrosis is a passive accidental cell death happening along with inflammatory cellular response caused by environmental disturbance (Figure 1.8) (Negroni *et al.*, 2015).

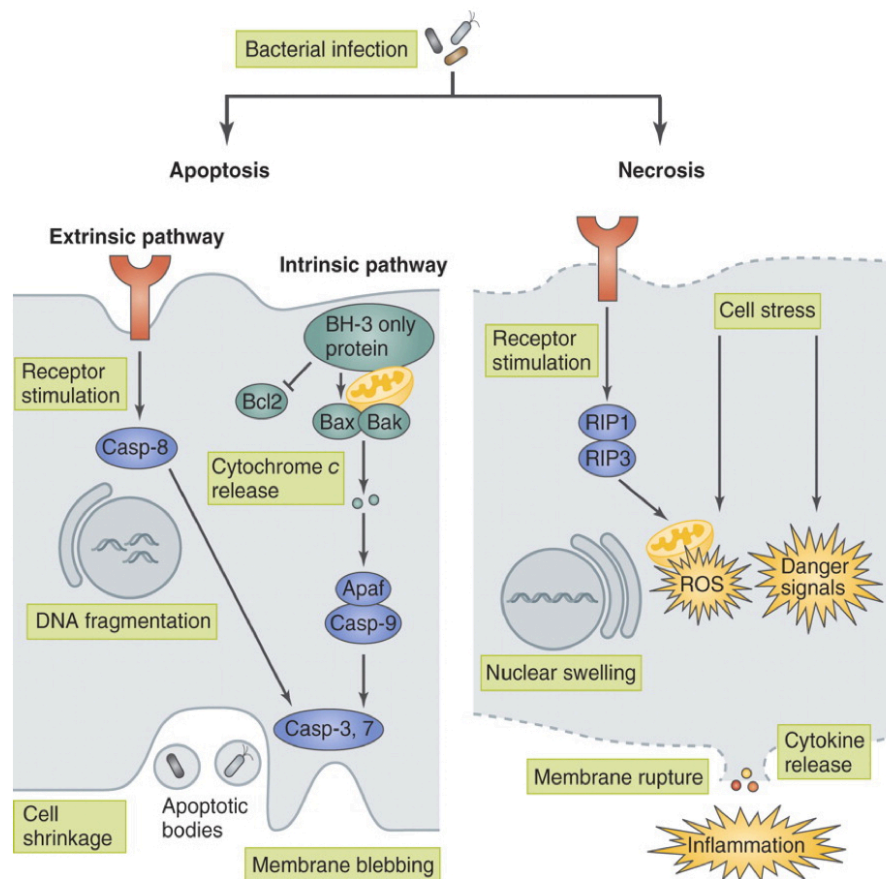


Figure 1.8 Cell death pathways induced by bacterial infection.

Bacteria have the ability to induce host cell death via different mechanisms including apoptosis and necrosis. Apoptosis can be stimulated by two different pathways, the intrinsic (mitochondria-mediated) pathway and extrinsic (receptor-mediated) pathway. All caspases are activated in apoptosis except caspase-1. At the morphological level, apoptosis can be characterised by membrane blebbing, cell shrinkage, DNA fragmentation and mitochondrial permeability. Then, apoptotic bodies start to appear containing bacteria which are engulfed by macrophages. Necrosis is type of inflammatory cell death which can be morphologically characterised by membrane rupture, nuclear swelling, and the release of cellular contents. Necrosis occurs by producing ROS or danger signals, such as lysosomal destabilization, calpain release, and depletion of ATP. These actions are induced by bacterial infection or physical damage. Figure adapted from (Ashida *et al.*, 2011).

Apoptosis is considered as a general pathway to remove old and infected cells. It is also involves the replacement of dead cells with new ones via mitosis to keep the stability of the cell population within the tissue. Therefore, cells are genetically programmed to contain the metabolic elements responsible to activate cell death (Ashida *et al.*, 2011). Moreover, cells swell during the necrosis process and then the plasma membrane is destroyed which leads to cell lysis. In contrast, cells shrink and identical DNA condensation occurs firstly within the apoptosis process, then the cell contents and damaged nuclei appear enclosed in the apoptotic bodies. The apoptotic bodies get phagocytised by phagocytes, however if they are not phagocytised, the content would lead to necrosis (Elmore, 2007).

1.3.5.2 Mechanisms of host cell death induced by bacterial infections

The interaction between eukaryotic host cells and prokaryotic cells (bacteria) is a complex process which involves different factors including bacterial virulence factors and cell innate immune defence factors (Lin *et al.*, 2010). Apoptosis is not the only process related to the stability of the immune system and renewing the functionally faulty cells. It also has a role in the immune response to infections. As well as that, intracellular pathogens may be killed by the apoptosis process. However, pathogens have developed different strategies to protect themselves against the host immune response. One of these strategies is to enhance apoptosis in immune cells such as T and B cells, neutrophils and phagocytes, thus pathogens secure their ability to survive, replicate and cause the infection. For example, exotoxin T produced by *P. aeruginosa* interferes with phagocyte functions (Galle *et al.*, 2012a, 2012b). Moreover, alpha toxin produced by *S. aureus* is able to induce the apoptosis in T cells by making pores in its membrane (Hossain, 2012). Furthermore, these toxins have the ability to lyse host cells

which allows the intracellular bacteria to spread and invade other cells through apoptosis (Broquet and Asehnoune, 2015). That would cause significant damage in the extracellular matrix or cell plasma membrane of the host cells. This damage caused by bacterial toxins might occur through enzymatic or pore development (Hossain, 2012). Protists as well use the same strategy to cause apoptosis in lymphocytes, granulocytes and phagocytes by the extrinsic pathway (Sitjà-Bobadilla, 2008), which will be mentioned in chapter four.

Balamuthia mandrillaris is a Free-Living Amoeba (FLA) which cause skin lesions as well as a fatal disease called Granulomatous Amoebic Encephalitis (GAE) (Cabello-Vílchez *et al.*, 2014a). In addition, *Acanthamoeba* can cause the same disease through its haematogenous spread. It is believed that skin lesion is the main entry route for *Acanthamoeba* to enter blood stream and interact with the blood brain barrier (BBB) (D'Auria *et al.*, 2012)

1.4 *Acanthamoeba*

Acanthamoeba are FLA that can cause human infections such as keratitis and encephalitis. In the last two decades, *Acanthamoeba* species have become recognised as one of the important microbes. They could play an important role as carriers and reservoirs for prokaryotes.

1.4.1 History and classification

Protists are eukaryotic organisms, and amoebozoa is one of the largest group belong to them (Scamardella, 1999; Khan, 2001). Amoebae were considered for a while as harmless microorganisms in the environment. However, later on researchers confirmed that amoebae could cause a fatal disease to human and animals, such as *Acanthamoeba* Granulomatous Encephalitis (AGE) (Winiecka-Krusnell and Linder, 2001). In 1930, Castellani discovered amoeba as an eukaryotic cell culture contaminant from a fungus culture plate. In 1931, the genus *Acanthamoeba* was discovered by Volkonsky (Figure 1.9) (Khan, 2009).

In 1970s, *Acanthamoeba* was first established as a cause of human disease. *Acanthamoeba* cause two well studied diseases in human: AGE and amoebic keratitis (AK) (Marciano-Cabral and Cabral, 2003).

The pathogenic species of *Acanthamoeba* include *Acanthamoeba castellanii*, *Acanthamoeba polyphaga*, *Acanthamoeba culbertsoni*, *Acanthamoeba palestinensis*, *Acanthamoeba hatchetti*, *Acanthamoeba rhysodes*, *Acanthamoeba divionensis*, *Acanthamoeba lugdunensis*, and *Acanthamoeba griffini*.

In the past, *Acanthamoeba* were classified in different groups based on the morphology of their cysts (Pussard and Pons, 1977). They divided *Acanthamoeba* into three groups

based on the cyst size and number of arms within a single cyst. According to their study, Group I have large cysts with diameter at least $\geq 18 \mu\text{m}$; and the endocysts and ectocysts are widely separated. Species belonging to this group have between 6-14 arms. *Acanthamoeba* belonging to group II have cysts with diameter of $\leq 18 \mu\text{m}$, with an ovoid, stellate, globular, or polyhedral endocyst and thick or thin ectocyst, while *Acanthamoeba* which were classified as group III have cysts with diameters of $< 18 \mu\text{m}$ with globular or ovoid endocysts, and thin ectocysts (Khan, 2009).

However, the ionic concentration of the growth media can have an effect on the morphology of the cyst, which would cause a variation in this morphological characteristic (Sawyer, 1971; Stratford and Griffiths, 1978). As a result, the morphological taxonomy criteria have been challenged as a main classification method (Moura *et al.*, 1992). Isolation, cultivation and microscopy were the main methods of *Acanthamoeba* identification, but due to the difficulty of interpretation, it has now been suspended. The discovery of molecular techniques led to work in classification of the genus *Acanthamoeba* based on sequences of Diagnostic Fragment 3 (DF3) of 18S rDNA. The Polymerase Chain Reaction (PCR) is one of these techniques which is used to amplify a region of deoxyribonucleic acid (DNA). This sequence is highly conserved, precise, reliable and informative (Schroeder *et al.*, 2001). Sequences of both nuclear 18S rRNA gene (rDNA) and mitochondrial 16S rRNA gene (rDNA) are suitable to classify *Acanthamoeba* isolates (Gast *et al.*, 1996; Chung *et al.*, 1998; Khan, 2009). Based on 18S rDNA sequences, genus *Acanthamoeba* is divided into 20 different genotypes (T1-T20). In addition, T2/T6 and T4 genotypes are divided to different subgroups (Siddiqui and Khan, 2012; Fuerst, 2014; Corsaro *et al.*, 2015).

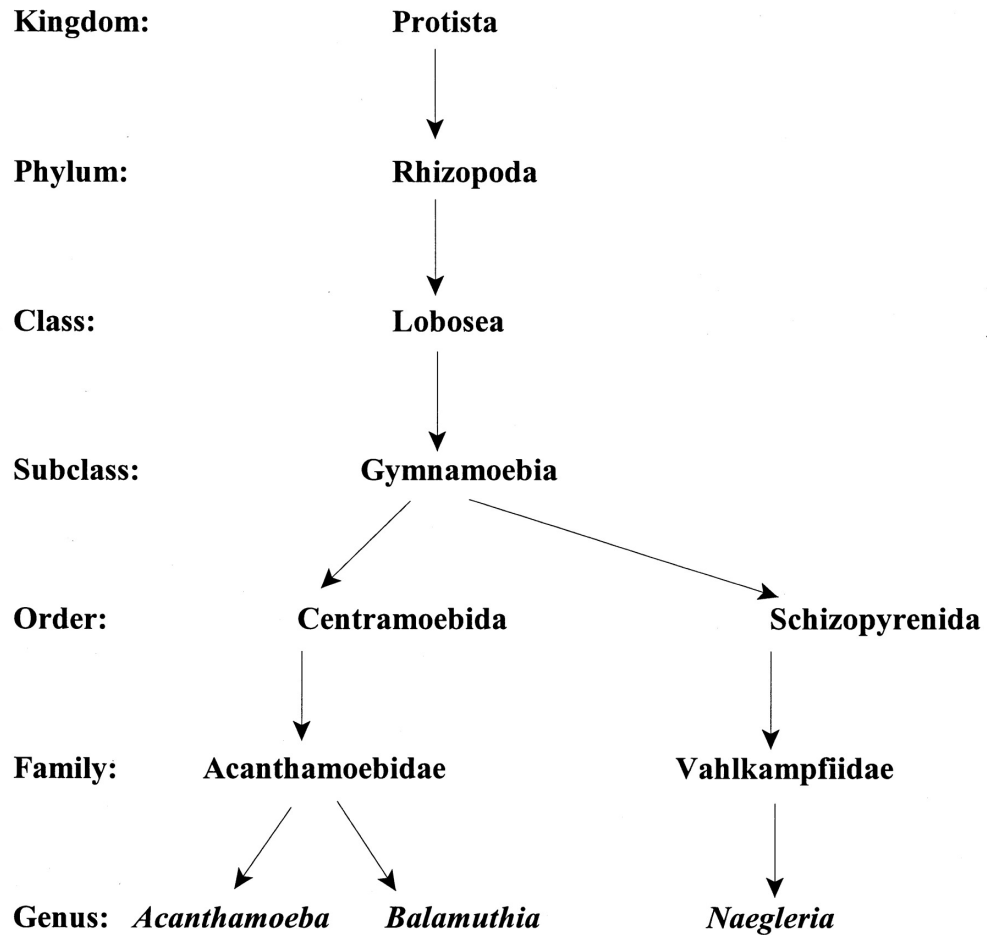


Figure 1.9 The classification scheme of protists, based on their genetic relatedness.

Figure adapted from (Marciano-Cabral and Cabral, 2003).

1.4.2 Ecology

Free-living amoebae are widely distributed in the environment, and they have been isolated from different sources, such as soil and air (Khan, 2009). *Acanthamoeba* can live in harsh conditions, such as frozen and salty environments (Dudley *et al.*, 2005). In addition, there are some virulence differences between *Acanthamoeba* isolated from different environments. For example, *Acanthamoeba* isolated from thermally-polluted factory discharges is more virulent than *Acanthamoeba* isolated from water surface. Furthermore, *Acanthamoeba* has been isolated from hospital settings and clinical samples, such as lung tissues, skin lesions, skin wounds and human faeces (De Jonckheere, 1991; Siddiqui and Khan, 2012).

1.4.3 Life cycle

Acanthamoeba has two main different stages in its life. The active stage of *Acanthamoeba* is called the trophozoite stage. The dormant stage of *Acanthamoeba* is the cyst stage. Growth conditions are very important in changing the stage of *Acanthamoeba* from active to dormant. The necessary conditions for trophozoites include an abundant food supply, temperature and neutral pH. The trophozoite varies in size from 25 to 40 μm and feeds on bacteria, algae, and yeast in the environment but also can exist axenically on nutrients in liquid taken up through pinocytosis. The reproduction of *Acanthamoeba* is asexual, that is it occurs via binary fission; and two new genetically identical *Acanthamoeba* will be the result of any division. This active stage contains the typical content of a eukaryotic cell, including cytoplasm, Golgi, free ribosomes and a nucleus. The trophozoite is characterized by the presence of acanthopodia, which are just found in the trophozoite stage but not in the cyst stage

(Siddiqui and Khan, 2012; Castrillón and Orozco, 2013). The cyst stage is characterized by the double walls called the endocyst and exocyst (Page, 1967).

The favourable conditions of pH, temperature, osmolarity and food are very important to keep the *Acanthamoeba* in the active stage; otherwise *Acanthamoeba* will change to be in a dormant stage, cyst (Figure 1.10) (Dudley *et al.*, 2005). The cyst stage can survive more than 20 years until exposed to a source of food when they excyst (Lloyd, 2014). The cyst stage is crucial in *Acanthamoeba* pathogenesis due to its ability to resist antimicrobial agents and physical treatments. Cysts can be airborne playing a significant role in *Acanthamoeba* distribution in the environment (Siddiqui and Khan, 2012; Lloyd, 2014).

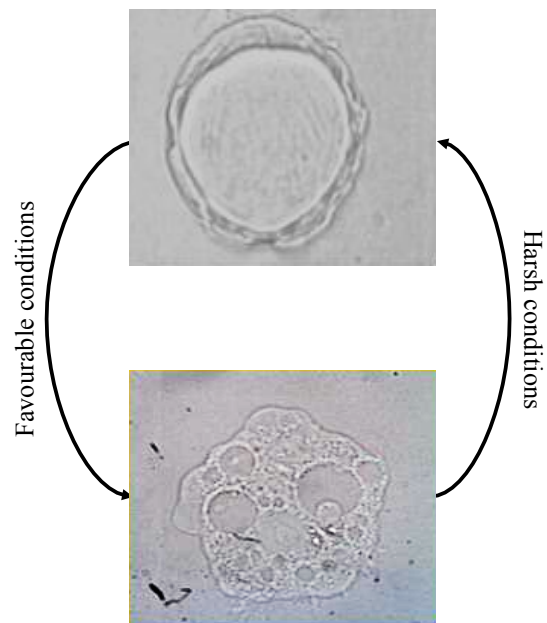


Figure 1.10 The life cycle of *Acanthamoeba* spp.

1.4.4 Feeding

Amoeba growth and survival depends on the amoeba feeding preferences (Bottone *et al.*, 1992, 1994) and bacterial density (Marciano-Cabral and Cabral, 2003). *Acanthamoeba* showed high amoebic yields upon digestion of low densities of Gram-negative bacteria. Examples of bacteria which serve as excellent food for *Acanthamoeba* include *Escherichia coli* K-12 (Huws *et al.*, 2005; Alsam *et al.*, 2006), and *Klebsiella pneumoniae* (Weekers *et al.*, 1993).

Acanthamoeba feeding occurs by phagocytosis and pinocytosis (Chambers and Thompson, 1976). Phagocytosis is a contact dependent process whereas pinocytosis is a non-binding process characterized by surface invaginations and vesiculations that takes up solutes and food particles (Bowers, 1980). Phagocytosis usually occurs by pseudopodia or by food cup formation. *Acanthamoeba* surrounds the food particles within its plasma membrane or pseudopodial projections into a membrane-enclosed particle that buds off into the cytoplasm. The membrane-enclosed particle called a phagosome subsequently fuses with a lysosome that contains a variety of hydrolytic enzymes (Alsam *et al.*, 2005a; Lorenzo-Morales *et al.*, 2015).

1.4.5 Diseases caused by *Acanthamoeba*

Acanthamoeba is a harmful pathogen that can cause fatal diseases, such as AGE, and corneal infection known as AK. Furthermore, *Acanthamoeba* could cause some infections (e.g. cutaneous, skin and bone infections) in immunosuppressed patients (Graffi *et al.*, 2013).

1.4.5.1 *Acanthamoeba granulomatous encephalitis (AGE)*

In 1972, AGE was identified as a human disease for the first time (Ringsted *et al.*, 1976). This disease mostly occurs in immunocompromised patients, such as HIV/AIDS and malnourished patients. AGE can be fatal and occurs in the central nervous system (CNS), where it may develop as a chronic disease spanning from several weeks to months. There are different entry routes for *Acanthamoeba* to cause this disease including lower respiratory tract, olfactory neuroepithelium, and skin lesions (Figure 1.11) (Garajová and Mrva, 2011). However, the incubation time of *Acanthamoeba* to cause AGE is still unknown, and it is assumed to be more than 10 days. Nowadays, the number of human immunodeficiency syndrome HIV/AIDS patients has increased; therefore, AGE has become a serious concern for human health (Figure 1.12) (Cuevas *et al.*, 2006).

1.4.5.1.1 Pathophysiology and treatment

There are many characteristic symptoms of AGE including confusion, stiff neck, headache, lethargy, ataxia, vomiting, aphasia, nausea, seizures, cranial nerve palsies, increased intracranial pressure, coma and death in the end (Bloch and Schuster, 2005). It is difficult to diagnose AGE, because of the similarities in its signs and symptoms with viral encephalitis and bacterial meningitis. However, scans by Magnetic Resonance Imaging (MRI) or cranial Computed Tomography (CT) may help in diagnoses and could reveal contrast enhancement areas indicating brain abscess or tumours (Figure 1.13) (Miltner and Bermudez, 2000).

There are some different drugs to treat AGE, such as trimethoprim-sulfamethoxazole, ketokonazole, amphotericin-B, rifampicin, fluconazole, sulfadiazine and albendazole (Kaushal *et al.*, 2008; Zamora *et al.*, 2014).

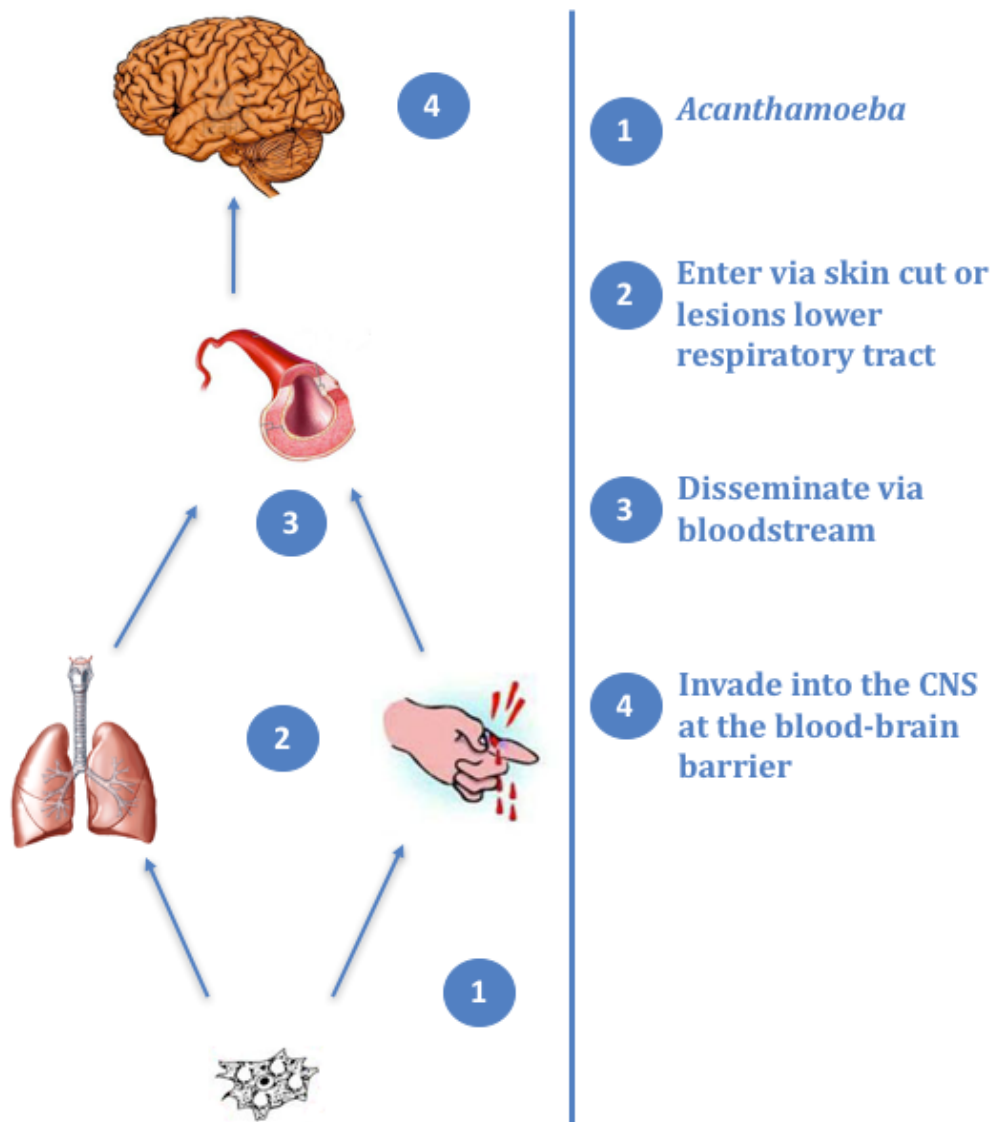


Figure 1.11 The haematogenous routes of *Acanthamoeba* entry into the CNS.

Acanthamoeba may pass through the lower respiratory tract or through skin injury then to blood brain barrier (BBB) via bloodstream.

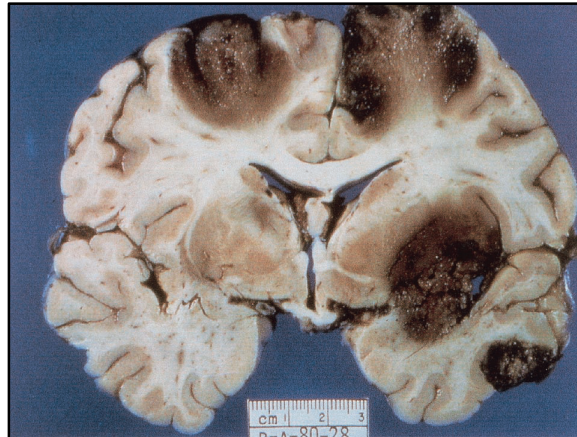


Figure 1.12 Coronal section.

Coronal section of the cerebral hemispheres with cortical and subcortical necrosis from a fatal human case of AGE (Marciano-Cabral and Cabral, 2003).

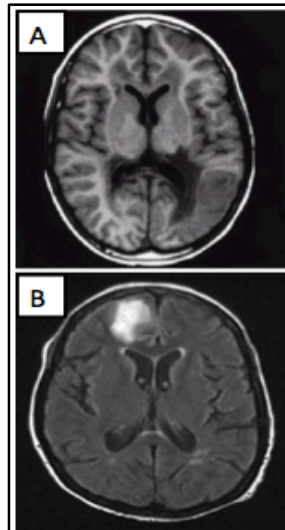


Figure 1.13 RMI of normal and infected brain with AGE.

(A) Normal brain MRI. (B) MRI of a patient's brain with AGE showing a hemorrhagic lesion in the right front lobe (Meersseman *et al.*, 2007).

1.4.5.1.2 Immunology of AGE

Haematogenous spread is a pre-requisite in AGE infections and complement activation is the inducer of the first line of defence. There are three pathways of complement activation, 1) classical pathway activated by antibody recognition of antigens on the surface of *Acanthamoeba*; 2) alternative pathway activated by C3 binding and auto-catalytically cleaved, then the attached C3b act as an opsonin; and 3) mannose-binding lectin bound by *Acanthamoeba* surface (Ferrante and Rowan-Kelly, 1983; Marciano-Cabral and Toney, 1998; Pumidonming *et al.*, 2011).

Normal human serum is effective against *Acanthamoeba* by activation of the alternative pathway of the complement system through the attachment of *Acanthamoeba* with C3 (Pumidonming *et al.*, 2011). Different proteins (C5, C6, C7, C8 and C9) in complement system in human body could kill *Acanthamoeba* by the damaging of amoeba membrane; or it could be killed by phagocytic cell following opsonisation by C3b (Ferrante and Rowan-Kelly, 1983). Overall, the immune system response against *Acanthamoeba* infections is not fully understood, and more studies are required to identify the factors that lead to this fatal infection (Marciano-Cabral and Cabral, 2003; Malla *et al.*, 2014).

1.4.5.2 *Acanthamoeba keratitis* (AK)

The reports showed that the first recognised cases of ocular infection were in 1973 in the UK and USA (Illingworth *et al.*, 1995), and the first case of AK was described in 1975 (Naginton *et al.*, 1974). There are eight species of *Acanthamoeba* which can cause AK, they are *Acanthamoeba castellanii*, *Acanthamoeba culbertsoni*, *Acanthamoeba hatchetii*, *Acanthamoeba polyphaga*, *Acanthamoeba rhysodes*, *Acanthamoeba lugdunensis*, *Acanthamoeba quina* and *Acanthamoeba griffini* (Logar and Kraut, 1997) (Ledee *et al.*, 1996; Omaña-Molina *et al.*, 2015). AK infection often affects the users of unsterile

solution for their contact lenses, and also affects the people who are swimming or bathing while wearing contact lenses (Ibrahim *et al.*, 2007). Moreover, this infection could affect non-lens users by exposure to contaminated water and soil (Dart *et al.*, 2009).

1.4.5.2.1 Pathophysiology and treatments

Acanthamoeba keratitis takes few days to several weeks to be cleared, but it depends on the route of amoeba's entry. The development of infection occurred by contaminated contact lenses is a much slower process, while it is rapid in the case of corneal trauma. The main symptoms of AK are represented by photophobia, pain, epithelial defects, and inflammation with redness and tearing that leads to epitheliopathy, perineural infiltrates, ring infiltrates, ulceration, abscess formation, hypopyon, scleritis, glaucoma, cataract, corneal perforation and posterior segment inflammation (Figure 1.14) (Alsam *et al.*, 2008; Lorenzo-Morales *et al.*, 2015).

A wide range of topical antimicrobial agents currently used for AK treatment. However, a combination of agents is used against cyst form as it is highly resistance to therapy. Low concentration of PolyHexaMethylene Biguanide (PHMB) is effective against both forms of *Acanthamoeba*, but is unfortunately toxic to human corneal cells. Chlorhexidine is an effective agent against both forms, and is not toxic on corneal epithelial cells. A combination of chlorhexidine, propamidine isethionate, dibromopropamidine, hexamidine and neomycin, have been used to treat AK, and is showing great results (Lorenzo-Morales *et al.*, 2015).

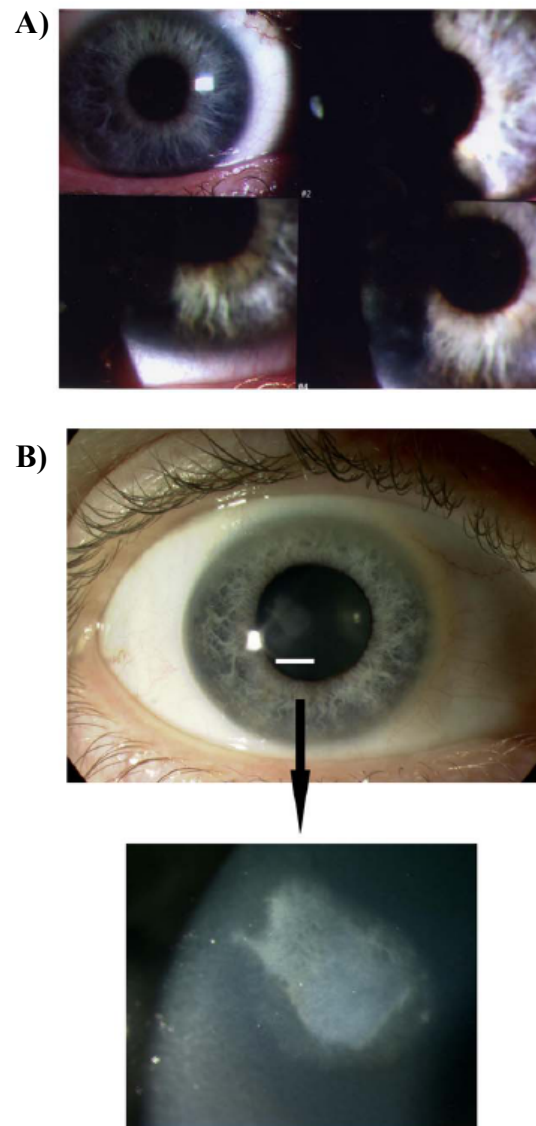


Figure 1.14 *Acanthamoeba*-infected eye.

(A) Early presentation of *Acanthamoeba* keratitis (B) Improvement of *Acanthamoeba* keratitis after 6 months' treatment with polyhexamethylene biguanide, propamidine-isethionate and neomycin. Figure adapted from (Alsam *et al.*, 2008).

1.4.5.2.2 Immunology of AK

The first defense line against AK is ocular surface, tear fluid and the eyelid. The tear fluid contains several compounds possessing antimicrobial and immunological properties (e.g. lysozyme, lactoferrin, β -lysins, secretory immunoglobulin A and prostaglandins). There are no significant effects of lysozyme and lactoferrin on *Acanthamoeba* binding to and cytotoxicity of human corneal epithelial cells (Dudley *et al.*, 2007; Alsam *et al.*, 2008).

The adherence of trophozoites to corneal epithelial cells is prevented by the conjunctiva being coated with secretory IgA. Previous studies proved that the mechanism of anti-*Acanthamoeba* IgA antibodies protect against AK by inhibiting the adherence of trophozoites to corneal epithelial cells without effecting the viability of *Acanthamoeba* trophozoites. Mannose-Induced Protein (MIP-133) is produced when trophozoites adhere to corneal epithelium (Hurt *et al.*, 2003). Repeated oral vaccination with both MIP-133 and cholera toxin elicit generation of IgA antibodies which block cytopathic effects in corneal cells *in vitro*, and that strongly supports its ability to be used as an anti-disease vaccine (Alizadeh *et al.*, 2005; Bouheraoua *et al.*, 2014; Lorenzo-Morales *et al.*, 2015).

Conjunctiva macrophage cells can be activated by interferon gamma (INF- γ) and lipopolysaccharide (LPS), and this activation could kill *Acanthamoeba* trophozoites (Alizadeh *et al.*, 2005).

1.4.6 *Acanthamoeba* pathogenesis

Several studies were conducted on the pathogenicity of *Acanthamoeba* infections. These studies were mainly conducted on the strains that cause corneal disease rather than that causing CNS disease. The pathogenicity of *Acanthamoeba* infections is a complex

process that involves many determinants grouped in contact-dependent and contact-independent factors (Baig, 2015; Lorenzo-Morales *et al.*, 2015).

1.4.6.1 Contact-dependent mechanisms

1.4.6.1.1 Binding

Binding of *Acanthamoeba* to the host cell is an important step in *Acanthamoeba* pathogenesis. Studies show that Mannose Binding Protein (MBP) is very important for *Acanthamoeba* to bind to both corneal epithelial cells and human brain microvascular endothelial cells, while mannose containing glycoproteins are present on the surface of corneal epithelium (Yang *et al.*, 1997; Shin *et al.*, 2001; Alsam *et al.*, 2003) (Panjwani, 2010; Yoo and Jung, 2012).

The transepithelial resistance of cell monolayers interacting with *Acanthamoeba* is reduced, which indicate the role of MBP as contact-dependent cytolysis (González-Robles *et al.*, 2006; Lorenzo-Morales *et al.*, 2015). As binding assays showed *Acanthamoeba* perfectly bind to collagen, laminin and fibronectin. *Acanthamoeba* invade and destruct host tissue after the association (Gordon *et al.*, 1993).

1.4.6.1.2 Phagocytosis

The pseudopodia of *Acanthamoeba* trophozoites are responsible of making contact with target cells in the presence of calcium channels and cytoskeletal elements. The binding of *Acanthamoeba* with epithelial cells is dependent on the induction of the host cell's signalling pathway and cytotoxic substances. Epithelial cells will be severely ulcerated and penetrated when incubating them together for 12 h at 35 °C. *Acanthamoeba* cause cytopathic effects in host cells through cytolysis and phagocytosis (Alsam *et al.*, 2005a; Siddiqui and Khan, 2012).

1.4.6.2 *Contact-independent mechanisms*

1.4.6.2.1 Extracellular proteases

Proteases such as, cysteine, serine and metalloprotease are produced by viruses, bacteria and protist. It is represented by cysteine, serine and metalloprotease. Pathogenic *Acanthamoeba* show high activity of extracellular protease. The relation between pathogenicity and the high activity of extracellular proteases propose that pathogenic *Acanthamoeba* use proteases to invade host cells. Some pathogenic *Acanthamoeba* spp. produce serine proteases, with range of molecular weights from 20 to 200 kDa (Alsam *et al.*, 2005b). Serine proteases are produced by *Acanthamoeba* trophozoites but not *Acanthamoeba* cysts. Proteases produced by *Acanthamoeba* has been identified as degraded enzyme which degrade fibronectin, fibrinogen, IgG, IgA, albumin, haemoglobin, protease inhibitors, IL-1, chemokines and cytokines. The main component of corneal stroma matrix is type I collagen, which can be degraded by *Acanthamoeba* proteases (e.g. serine, cysteine and metalloprotease). Moreover, the production of metalloprotease was shown to exhibit degradation of ECM (extracellular matrix) components such as collagen and laminin (Alizadeh *et al.*, 2007; Rocha-Azevedo *et al.*, 2010; Tripathi *et al.*, 2012).

1.4.6.2.2 Phospholipases

Phospholipases enzymes are classified into five major classes A1, A2, B, C and D, and their role is to catalyse the hydrolysis of phospholipids into fatty acids and other lipophilic substances. However, a few studies referred to the presence of phospholipases in *Acanthamoeba* infections. Overall, each of these factors presented have a crucial role in invasion of *Acanthamoeba* trophozoites and development of disease. However, their

precise roles remain to be determined in further studies (Khan, 2008; Lorenzo-Morales *et al.*, 2015).

1.4.7 Indirect virulence factors

Acanthamoeba ability to initiate any infection in the human body is a complicated process depending on direct and indirect factors. Some of these factors are *Acanthamoeba* cell morphology (e.g. the active stage, trophozoite), temperature (e.g. the suitable temperature for *Acanthamoeba* to grow from 12-37 °C), osmolarity ranging between 50-80 mosmol, pH ranging between 4-12, phenotype switching, drug resistance (*Acanthamoeba* elicit resistance against most of the drugs used), ubiquity (*Acanthamoeba* found in diverse environments), and biofilms (biofilms are suitable niches to provide nutrition for *Acanthamoeba*) (Alsam *et al.*, 2008; Khan, 2008).

1.5 Alternative therapeutic techniques

The increase in multidrug-resistant bacteria has been widely reported. Different resistance modes could be present in a bacterial strain, and that could be related to increasing virulence leading to increased morbidity and mortality in patients (Bax and Griffin, 2012). Several factors are associated with the increased antimicrobial resistance in bacteria, such as the overuse and improper uses of antimicrobial agents (Spellberg *et al.*, 2008; Juul *et al.*, 2016). This could happen through several mechanisms such as, some microorganisms develop the ability to neutralize the antibiotic, and others can change the target attack site, rendering the antibiotic ineffective. Mutations are not uncommon in bacterial genetic material and also, resistance could increase through the transfer of resistance determinants intra and inter species (Bozdogan *et al.*, 2003; Aminov and Mackie, 2007; Li and Nikaido, 2009; Wegener, 2012). MRSA and *P. aeruginosa* are just examples of antibiotic-resistant bacteria, which often cause wound infections. These antibiotic-resistant bacteria are difficult to treat, often associated with prolonged hospital stay, and they could cause complications and can increase the risk of death (Sawa *et al.*, 2014; Duerden *et al.*, 2015). In addition, *Acanthamoeba* have two stages in their life cycle, trophozoite and cyst forms. *Acanthamoeba* trophozoites turn to cyst in harsh conditions notably, presence of medications. Some studies showed that bacteria have the ability to survive and multiply inside both forms of *Acanthamoeba* (Alsam *et al.*, 2006; Thomas *et al.*, 2010; Cardas *et al.*, 2012). Other studies concluded that the protected bacteria inside *Acanthamoeba* become more resistant to antibiotics (Spellberg *et al.*, 2008). With this increased resistance, the need for new antimicrobial agents (e.g. iron chelators) is urgent (Lamb, 2015; Rangel-Vega *et al.*, 2015).

1.5.1 Importance of iron for pathogens

Iron is one of the essential elements required for the growth of all bacteria (Lewin, 1984). Iron has many different functions in bacterial cells. It has an effect on the synthesis of the cell structures, proteins, enzymes, DNA and RNA, in addition to its influence on the metabolism process. Those functions, also showed the main important role of iron for the bacteria to enhance the interaction of bacteria with the host cells which would include the pathogenicity (see table 1.3) (Messenger and Barclay, 1983; Yamaji *et al.*, 2004).

Affected Area	Effects
Cell structure and contents	Shortage of iron can cause: growth inhibition, decrease in RNA and DNA synthesis, changes in morphology of cells
Metabolism and Metabolic products	Examples of metabolism processes requiring iron: tricarboxylic acid cycle, oxidative phosphorylation, nitrogen fixation, aromatic biosynthesis Some of metabolic products which regulated by increased or decreased the iron element: porphyrins, toxins, hydroxamates, cytochromes, siderophores, aromatic compounds, DNA and RNA
Proteins and enzymes	Examples of proteins and enzymes required iron: Peroxidase, superoxide dismutase, nitrogenase, hydrogenase, glutamate

Table 1.3 Role of iron in microorganisms.

Table adapted from (Messenger and Barclay, 1983).

Iron roles are considered to be catalytic rather than regulatory roles. Iron is required to activate the responsible enzymes for the oxidative metabolism of the tricarboxylic acid cycle, such as aconitase, which is an iron-sulphur protein that catalyses the interconversion of citrate and isocitrate. Although iron is important for the metabolism through tricarboxylic acid cycle, the aerobic and facultative anaerobic bacteria are able to metabolise through glycolysis using glycolytic enzymes which do not require iron element (Braun and Hantke, 2011; Ledala *et al.*, 2014). As a result, phosphorylation would be the main source of energy rather than oxidative phosphorylation. However, the shortage of iron would lead to reduced DNA and RNA levels in bacteria. The DNA synthesis will fail as a result of the dysfunction of ribonucleoside diphosphate which is responsible for deoxyribonucleotide synthesis (Hammer and Skaar, 2011). Moreover, the deficiency in the amount of iron could make a significant change in the structure of tRNA. Some of nitrogen assimilation stages need iron as an essential element. For example, nitrogenase enzyme in bacteria consists of an iron-molybdenum protein and an iron-protein (Miller *et al.*, 2009). The intracellular iron in the host cells is not available for the extracellular bacteria. In addition, the amount of free iron in the serum is very low, as it is conjugated with high affinity iron-binding proteins. Therefore, conjugated iron in the host is a natural immunity of the host to limit the growth of pathogens. This limitation of iron leads the microorganisms to improve their strategies to obtain the iron required for growth and survival within the host body (Crosa and Payne, 2004; Cassat and Skaar, 2013; Ledala *et al.*, 2014).

1.5.2 Mechanisms of iron acquisition

The iron in form of Fe^{3+} is almost insoluble, but it is toxic with the formation of oxygen radicals which could damage the DNA and proteins. Iron is mostly found as bound

element with proteins such as transferrin, lactoferrin and hemoglobin. To get the iron they need, microorganisms have developed several mechanisms. They can use their own siderophores or other siderophores of other microorganisms to release the iron compound and then transport the iron into their cytoplasm through specific transport systems. Or, they can get the iron from the host heme in blood and iron binding proteins. They can deal with the shortage of iron via enhancing gene transcription encoding iron-transport proteins. They are able to synthesize different iron transport systems according to iron available in any infection of the human body (Hider and Kong, 2010).

1.5.2.1 Iron acquisition in *S. aureus*

Staphylococcus aureus responds to the shortage of iron in the host by activating the ferric uptake regulator (Fur). In most Gram-negative bacteria, Fur regulates iron metabolism to precisely control cytoplasmic iron levels. Although Fur regulation involves the binding of Fur-Fe²⁺ to the promoter region as a repressor and can function as an activator even without an iron cofactor in some pathogenic bacteria (Deng *et al.*, 2012). Staphylococci obtain the iron through siderophores and heme acquisition systems (Torres *et al.*, 2010). There are two types of carboxylate siderophores in *S. aureus* such as, staphyloferrin A and staphyloferrin B. Staphyloferrins are characterised by two receptors, HtsA specific for staphyloferrin A and SirA, specific for staphyloferrin B (Cheung *et al.*, 2009; Cotton *et al.*, 2009; Grigg *et al.*, 2010). In addition, *S. aureus* has another receptor for iron known as Fhu transporter, which is responsible for transferring Fe³⁺ hydroxamates to inside bacteria (Beasley *et al.*, 2011; Hammer and Skaar, 2011). Furthermore, *S. aureus* secretes haemolysins which lyse red blood cells resulting in acquisition of the hemoglobin. This is followed by a sequence of steps to extract the heme from hemoglobin by using some proteins in the *S. aureus* cell

wall and plasma membrane ending with internalization of heme by using the Isd system. Two receptors within the Isd system are colocalized on the cell surface and both are important for the virulence of *S. aureus*, namely IsdB (haemoglobin receptor) and IsdA (heme receptor). In the cytoplasm, *S. aureus* produces a heme-oxygenase enzyme (staphylobilin), which extract the iron from the porphyrin ring by degrading the heme via IsdG and IsdI (Mazmanian *et al.*, 2003; Muryoi *et al.*, 2008; Hammer and Skaar, 2011).

1.5.2.2 Iron acquisition in *P. aeruginosa*

There are four different strategies of iron uptake used by *P. aeruginosa*. The first and main strategy is via siderophore production, including pyoverdine and pyochelin, and through TonB-dependent receptors (TBDR) in the cytoplasmic membrane protein (Schalk *et al.*, 2012; Schalk and Guillon, 2013). *P. aeruginosa* pyoverdine is a high affinity siderophore which is needed in the case of acute infections. Pyoverdine is a complex siderophore containing a chromophore and a peptide chain. This type of siderophore is characterised by fluorescent pigment produced by grown *P. aeruginosa* in low iron conditions (Visca *et al.*, 2007). Pyoverdines have the ability to separate iron from transferrin, and their production is required to cause infection in a burnt mouse model (Imperi *et al.*, 2013), whereas, TonB protein mutation is avirulent in this mouse model (Takase *et al.*, 2000). Along with the pyoverdine role as a siderophore, it acts also in molecular signalling to enhance two extracellular virulence factors, the protease PrpL and the exotoxin A (Cornelis, 2010). The other siderophore of *P. aeruginosa* is pyochelin which has a very low affinity for iron, and can be produced by all strains of *P. aeruginosa* (Brandel *et al.*, 2012). Pyochelin is firstly produced by *P. aeruginosa* however, in the case of a shortage of iron, bacteria switch into pyoverdine production

(Dumas *et al.*, 2013). Pyochelin can be found in chronic infections, as it plays a role in the inflammatory response by damaging the host tissues (Lyczak *et al.*, 2002).

Furthermore, *P. aeruginosa* use Has and Phu systems to uptake the heme from the hemoproteins (Ochsner *et al.*, 2000). *P. aeruginosa* Has system secretes heme-binding hemophore protein in order to extract the heme from the hemoproteins such as hemoglobin, while the heme is extracted directly in the Phu system via an outer membrane TBDR. In order to internalize the heme, periplasmic binding protein will bind with heme and transport it to the cytoplasm via ABC transporter followed by degrading the heme by heme oxygenase to extract the iron (Barker *et al.*, 2012; O'Neill *et al.*, 2012).

Other strategy used by *P. aeruginosa* to obtain iron is xenosiderophore. The ability of *P. aeruginosa* to use several different types of xenosiderophores could help it to compete with other microorganisms to uptake the available iron. However, the importance of xenosiderophore iron-uptake strategies in infections has not been determined. They could be important in the case of polymicrobial infections, by making *P. aeruginosa* have an advantage in the presence of other microbes at the site of infection. *P. aeruginosa* has the ability to take up siderophores produced by other microorganisms (siderophore piracy) (Traxler *et al.*, 2012; Cornelis and Dingemans, 2013).

1.5.2.3 Iron acquisition in *Acanthamoeba*

A few reports have described the mechanisms of iron-uptake by the protozoa and the role of protozoan proteases in the degradation of iron-binding proteins. However, one of the main pathogenicity factors of *Acanthamoeba* is protease production. *Acanthamoeba* like other organisms need iron to survive and multiply (Siddiqui and Khan, 2012).

Proteases have been determined as factors used by protozoa to degrade iron-binding proteins in the host, such as hololactoferrin, transferrin, and hemoglobin (Serrano-Luna *et al.*, 1998; León-Sicaireos *et al.*, 2005; Reyes-López *et al.*, 2011). Cysteine proteases produced by *Acanthamoeba castellanii* and other protozoa have been suggested to be the responsible factor in degrading iron-binding proteins. These proteases could be involved in iron uptake and may represent a virulence factor to initiate different diseases caused by *Acanthamoeba* (Reyes-López *et al.*, 2011; Martínez-Castillo *et al.*, 2015; Ramírez-Rico *et al.*, 2015). Similar to the mammalian cells, transferrin receptors (Tfr) in the protozoa membranes are responsible for the transport and uptake of iron by endocytosis, however, there is little information about the signals and pathways induced by this mechanism to obtain iron (Reyes-López *et al.*, 2015).

1.5.3 Iron chelation techniques

The aim of chelation therapy is to reduce the iron level in the area. Chelation therapy has been established to treat patients with thalassemia in the 1980's (Borgna-Pignatti *et al.*, 2004). The aim of using this therapy in thalassemia patients is to remove the overload of iron and therefore reduce the complications related to iron burden. Many iron chelators have been reported in the literature, such as deferoxamine (DFO) and deferiprone (DFP) (Poggiali *et al.*, 2012).

DFP is one of hydroxypyridinone iron chelators synthesized by Dr. Kontoghiorghes in the early to mid 1980s in the laboratory of Professor R. Hider at the University of Essex, United Kingdom (Kontoghiorghes, 1985). The medicinal chemists in this laboratory were researching a molecule that could be taken orally, bind iron in conditions of iron overload, such as thalassemia, and excrete it from the body (Victor Hoffbrand, 2005). Moreover, the excitement over the discovery of a potentially

effective oral iron chelator led the investigators to initiate a development program of animal studies that would lead them to the most rapid route to a trial in humans. The first publication of the use of DFP in man was published in 1987 (Kontoghiorghes *et al.*, 1987).

The affinity of a selective iron chelator is very important when the high-affinity ones can easily be taken up by microbes. So, the affinity of iron chelator agents must be very high, enabling them to compete efficiently with siderophores. Moreover, topical application of chelators may be used in different ways such as cosmetic application, or in wound healing and in the treatment of wound infections (Qiu *et al.*, 2011).

There are some recent studies showing the antimicrobial effect of DFP, DFO and other developed iron chelators on microorganisms, such as *E. coli*, *S. aureus* and *P. aeruginosa* (Qiu *et al.*, 2011; Xu *et al.*, 2011; Thompson *et al.*, 2012; Zhou *et al.*, 2015).

1.6 Aim and objective(s)

MRSA and *P. aeruginosa* are the main multi-drug resistant pathogens associated with deep wound infections and then septicaemia. Treatment is problematic and re-infection is quite common. As well as bacteria, wounds can be colonised with protozoan microorganisms such as *Acanthamoeba* which feed on bacteria. The wide distribution of *Acanthamoeba* in the environment and its role as a Trojan horse for different bacterial strains suggest they may play an important role in wounds, acting as a protector for the intracellular bacteria against the treatment. As well as that, episodes of wound infection and healing periods could be attributed to the presence of *Acanthamoeba*. However, there is no single report showing the presence of *Acanthamoeba* in infected wounds. The main questions of this thesis are: does *Acanthamoeba* occur in wounds and does it have any role in wound infections?

To this end, many aspects and objectives have been investigated.

- 1- The ability of MRSA and *P. aeruginosa* strains to interact with and survive inside a human skin cell line and in different *Acanthamoeba* strains.
- 2- To examine new alternative therapies against these multi-drug resistant microorganisms.
- 3- To study the role and pathway of these microorganisms in inducing cell death by using the human skin cell line and stem cells that can be used to speed up wound healing.
- 4- To look for the presence of *Acanthamoeba* in clinical samples obtained from infected wounds.

Chapter Two

2

The interaction of MRSA and *Pseudomonas* with skin epithelial cells and *Acanthamoeba* in wound infections

Chapter 2 : The interaction of MRSA and *Pseudomonas* with skin epithelial cells and *Acanthamoeba* in wound infections

2.1 Introduction

Wound infections are the second common amongst health care associated infections such as urinary tract infection in hospitals, accounting for 10–16 % of all nosocomial infections (Kingsley, 2001; Frescos and Rando, 2008). There are many factors that could affect the progress of wound infections and make them more complicated. Large numbers of different microorganisms will enter the wound and the inflammatory response will result, as the integrity and protective function of the skin is breached. That would be clear and easy to diagnose by the classic signs of inflammation, such as redness, pain, fever and swelling. The process of the body's response is to restore the stability of the interdependence of the elements to return homeostasis in the infected area (Calvin, 1998; Sibbald *et al.*, 2003).

All wounds are colonised with different types of microbes (Bryant and Nix, 2012). In fact, these microorganisms are not all harmful and they are naturally found as flora on the skin's surface. Although the intact skin acts as a natural barrier against these microbes, a wound can significantly compromise this defence mechanism and allow microbes to enter and cause infections. The target layer of the skin for pathogenic microbes to cause infection is the underlying tissue, where there are suitable conditions and nutrients for microbes to colonise, grow and multiply. These microorganisms include bacteria, fungi, protist and viruses. MRSA and *P. aeruginosa* are the most common causes of wound infections and are isolated from both chronic and acute wounds (Gardner *et al.*, 2001). There are some studies which show their prevalence in different wound sites, such as surgical sites, penetrating trauma, burn wounds and ulcers (Gomez *et al.*, 2009; Keen *et al.*, 2010). The presence of bacteria in chronic and non-

healing wounds is to some extent high. Other studies show that the presence of MRSA and *P. aeruginosa* as polymicrobial infection in wound can lead to increased antimicrobial resistance (Pfaller *et al.*, 1989). Therefore, MRSA and *P. aeruginosa* were selected for this study.

2.1.1 MRSA

MRSA is a common opportunistic pathogen, which causes the majority superficial skin infections, thus increasing morbidity and mortality. MRSA-associated infections in both acute and chronic wounds have been reported and well-documented. Virulence factors and toxins produced by MRSA play a main role in its pathogenicity. These virulence factors include α -hemolysin (Hla), staphylococcal protein A (spa), and Panton-Valentine leukocidin (PVL). Surface proteins of MRSA have multi functions in the pathogenesis. In addition to their functions in the metabolism of the bacterial cell wall, they have a main role in the binding to the host cells and biofilm formation (Foster and Höök, 1998; Pastar *et al.*, 2013). MRSA surface binding proteins that interact with human skin matrix molecules may play a role in the MRSA colonisation and then infection, especially when the matrix proteins are exposed through breaching the superficial tissue layers. Together, *S. aureus* proteins G and X (SasG, SasX) and clumping factor B has been shown to have a role in binding to nasal epithelial cells (O'Brien *et al.*, 2002; Roche *et al.*, 2003; Corrigan *et al.*, 2009). SasX protein has been shown as an important binding protein, as it is linked to an MRSA epidemic. This protein is encoded on a Mobile Genetic Element (MGE) that often occurs in ST239 MRSA strains, which are considered as the common source of MRSA infection in Asia (Li *et al.*, 2012).

2.1.2 *Pseudomonas aeruginosa*

P. aeruginosa is another pathogen, which can cause serious nosocomial infections, such as surgical-site infection that could develop to be a chronic wound infection. Similar to

other pathogens, cellular and extracellular virulence factors (e.g. exoenzyme S) play a role in the pathogenesis. These factors help the microorganism to colonise, invade then survive inside the host cells (Rocha *et al.*, 2003; Harmsen *et al.*, 2010). *P. aeruginosa* virulence factors could be classified into two main types. Firstly, virulence factors within acute infections, which could be either surface proteins (e.g. pili) or secreted enzymes (e.g. exotoxin A) (Ben *et al.*, 2011). Pili are one of these surface factors which are responsible for the binding of bacteria to host epithelial cells (Hahn, 1997). Exoenzyme S (ExoS) is another factor that reinforces adhesion to epithelial cells via disruption of normal cytoskeleton. In addition, ExoS motivates ADP-ribosyltransferase activity of Ras family proteins. Expression of the ADP-ribosyltransferase domain also inhibits *Pseudomonas* internalization by epithelial cells and macrophages (Shaver and Hauser, 2004). Secondly, virulence factors occur within chronic infections, such as pyoverdine, which helps the bacteria to survive and multiply despite the limitation of iron in the environment. Pyoverdine is a small molecule iron chelator, which is necessary for the metabolism and regulation of the secretion of some virulence factors (exotoxin A, proteases). In addition, the Quorum Sensing system (QS) is one of the regulation systems which control the expression of virulence factors (Ben *et al.*, 2011). There are two types of QS population density-dependent system which are expressed in *P. aeruginosa*: LasI-LasR and RhII-RhlR. Both systems have a role in pathogenicity by regulating the expression of virulence factors; such as exotoxin A (ToxA) and ExoS which could induce apoptosis in lymphocytes (Pastar *et al.*, 2013).

2.1.3 *Acanthamoeba*

The wide distribution of *Acanthamoeba* in the environment gives a high risk of this organism using deep wounds as a gateway to the human body to go through the blood stream and then to the Blood Brain Barrier (BBB), potentially causing AGE in

immunocompromised patients. However, there are no reports to show the presence of *Acanthamoeba* in infected wounds in healthy individuals. The presence of *Acanthamoeba* in infected wounds will be discussed in detail in (Chapter 5). Some studies have shown that *Acanthamoeba* is the Trojan horse for a variety of microbes including MRSA and *P. aeruginosa* as they can survive and multiply inside both *Acanthamoeba* trophozoite and cyst stages, then burst the *Acanthamoeba* (Cardas *et al.*, 2012; Yousuf *et al.*, 2013). Hence, three *Acanthamoeba* strains were selected to perform this study.

Here, the ability of MRSA and *Pseudomonas* strains isolated from wound infection to bind with, invade, survive and multiply inside human skin cell line and different pathogenic and non-pathogenic strains of *Acanthamoeba* were investigated.

2.2 Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

2.2.1 Cell lines and cell culture

Human KB epithelial cells isolated from human epidermal carcinoma of the mouth were kindly provided by the University College of London (UCL). KB epithelial cell line was provided as proliferating cells in T-25 flask. Cells were cultured and maintained in Eagle's Minimum Essential Medium (EMEM) supplemented with 10 % (v/v) foetal calf serum, 0.1 % (w/v) (1000 U/mL, 1 mg/mL) penicillin/streptomycin and 1 % non-essential amino acids. Cells were transferred into three T25 flasks and incubated at 37 °C in humidified 95 % air, 5 % CO₂. Theoretically, cells become confluent within 2 days (~3x10⁶ cells). All transfers, used in experiments, were between transfer numbers 4 to 10.

2.2.1.1 *Transfer cells into T25 flask*

To transfer cells, growth medium was aspirated off from the confluent flask. Growing cells were washed by phosphate-buffered saline (PBS) once. Then, 0.5 mL Trypsin-Ethylenediaminetetraacetic acid (EDTA) (1X) was added into the tissue culture flask and rocked them for 1-2 min, and then the flask was tapped. Once the cells detached from the tissue culture flask, the trypsin was immediately inactivated by adding 5 mL of growth medium. The flask contents were transferred to a 15 mL centrifuge tube. The sample was centrifuged for 4 min at 1000 x g. The supernatant was discarded and the pellet was re-suspended in warm growth medium. In the end, the suspended cells were transferred to three other flasks and incubated at 37 °C in humidified 95 % air, 5 % CO₂ incubator.

2.2.1.2 Seeding cells in 24 well-plate

The cells were detached as described earlier. The suspended cells were seeded in 24-well plate ($\sim 5 \times 10^5$ cells per 0.5 mL in each well) and incubated at 37 °C in humidified 95 % air, 5 % CO₂ incubator. and isolated by Dr J Lorenzo-Morales

2.2.2 Acanthamoeba culture

Two clinical isolates of *Acanthamoeba castellanii* (T1 and T4 genotype), isolated from granulomatous encephalitis and keratitis patient, and one environmental isolate (T7 genotype) used in the study were kindly provided by Dr S Maciver, and isolated by Dr J Lorenzo-Morales (University of Edinburgh). The amoebae were grown in 10 mL Peptone Yeast Glucose (PYG) medium (0.75 %, w/v, proteose peptone; 0.75 %, w/v, yeast extract; 1.5 %, w/v, glucose) in T-75 tissue-culture flasks at 30 °C (Khan 2001, Alsam *et al.* 2006). The medium was refreshed every 17–20 h prior to experiments to get 95 % confluence in the trophozoite form.

2.2.3 Bacterial culture

Three MRSA strains and three *Pseudomonas* strains were used in the present study. All bacterial strains were obtained from Colchester General Hospital by Dr Tony Elston. These strains are clinical isolates from wound infection patients. All strains were streaked onto nutrient agar and incubated overnight in 37 °C air incubator, and refreshed every week. One or two single colonies of bacteria were grown in Luria-Bertani (LB) broth overnight at 37 °C in air incubator. The MRSA strains concentration were estimated by Optical Density (OD) measured by spectrophotometer [Cecil CE 2041] at wavelength 595 n.m. multiplied by a factor of 0.22 ($OD_{595} 0.22 = \sim 10^8$ CFU/mL), while *Pseudomonas* strains were measured with OD 0.35 ($OD_{595} 0.35 = \sim 10^8$ CFU/mL) at the same wavelength.

2.2.4 Association assays

This assay was used to study the interaction of bacterial strains with the human skin cell line and *Acanthamoeba* (Figure 2.1). In brief, human cells or *Acanthamoeba* were cultured and grown in 24-well plates in their own medium [EMEM for KB epithelial cells and PYG for *Acanthamoeba*] ($\sim 5 \times 10^5$ cells per 0.5 mL in each well); next, plates were incubated till confluency. Then, the growth medium was discarded, and Roswell Park Memorial Institute medium 1640 (RPMI 1640) (with Stable Glutamine and Phenol Red) was added for *Acanthamoeba*, and EMEM medium for KB epithelial cell line. Next, bacteria ($\sim 5 \times 10^6$ CFU/well) were added onto cells with medium and incubated for 1 h at room temperature for *Acanthamoeba*, while KB epithelial cells with bacteria were incubated 1 h at 37 °C in humidified 95 % air, 5 % CO₂ incubator. Then, supernatants were discarded and washed by PBS once to remove non-adherent bacteria. Next, *Acanthamoeba* were lysed by adding 0.5 % (v/v) sodium dodecyl sulfate (SDS) final concentration with PBS for 20 min, while KB epithelial cell line was lysed by 0.5 mL of CellLytic M. In the end, 20 µl of lysed solution was cultured onto Cysteine lactose electrolyte deficient (CLED) agar, and incubated overnight at 37 °C in air incubator. Then number of recovered bacteria were counted. The bacteria associated with epithelial cell lines or *Acanthamoeba* were calculated as follows: number of bacteria recovered (CFU) / number of bacteria inoculated at the start of the experiment $\times 100 =$ % of bacteria associated with cells (Alsam *et al.*, 2006; Yousuf *et al.*, 2013).

2.2.5 Invasion assays

To determine the ability of bacteria to invade or be taken up by *A. castellanii* and the KB epithelial cell line, invasion assays were performed (Figure 2.1). Briefly, *Acanthamoeba* or epithelial cells were grown until confluent in 24-well plates as described above. After 1 h incubation with bacteria as detailed above, the wells were

washed with PBS, and then antibiotics were added to kill extracellular bacteria (vancomycin against MRSA, and ciprofloxacin against *Pseudomonas*; 100 µg/mL final concentration, for 45 min). Finally, cells were washed lysed and then cultured onto CLED agar and then, bacteria were counted as described above. The intracellular bacteria were calculated as follows: number of bacteria invade each cell or amoeba = number of bacteria recovered (CFU) / number of bacteria inoculated at the start of the experiment $\times 100$ = % of intracellular bacteria inside cells (Alsam *et al.*, 2006; Yousuf *et al.*, 2013).

2.2.6 Intracellular survival assays

To determine the long-term fate of bacteria inside *A. castellanii* and KB epithelial cell line, intracellular survival assays were performed (Figure 2.1). Briefly, *Acanthamoeba* or epithelial cell line were incubated with bacteria, followed by the addition of antibiotic for 45 min, as described above. After incubation, wells were washed with PBS. Then, *Acanthamoeba* were incubated in 0.5 mL RPMI 1640 for overnight at 30 °C, while epithelial cell line was incubated in 0.5 mL EMEM medium for overnight at 37 °C in humidified 95 % air, 5 % CO₂ incubator. Finally, *Acanthamoeba* or epithelial cell line were lysed and cultured onto CLED agar and intracellular bacteria were counted as described above. The survival of intracellular bacteria after 24 h incubations were calculated as follows: number of bacteria recovered (CFU) / number of bacteria inoculated at the start of the experiment $\times 100$ = % of intracellular survival bacteria inside cells (Alsam *et al.*, 2006; Yousuf *et al.*, 2013).

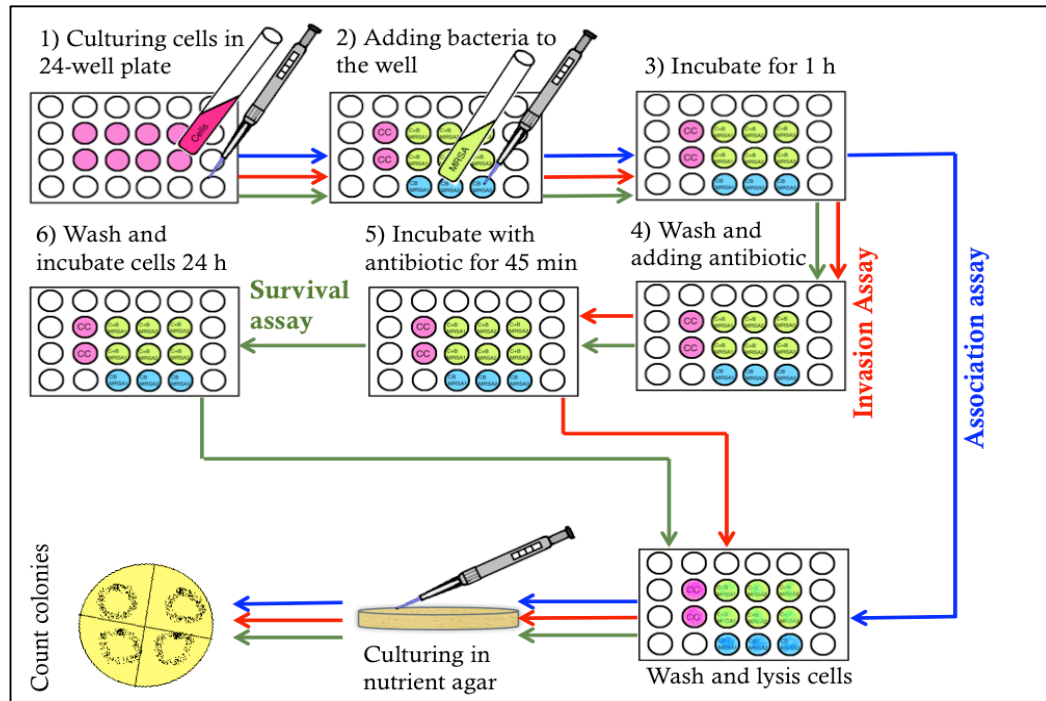


Figure 2.1 Stages of interaction assays.

Stages of interaction assays (association, invasion and survival) of bacterial strains with host cells (*A. castellanii* or epithelial cells).

2.2.7 Software and statistics:

Microsoft Office Excel programme was used to prepare graphs and to calculate the means, standard deviation and standard error. To find the significant differences between bacterial strains, two-tail t-Test was applied to calculate *P* value.

2.3 Results

Association assays were performed to determine the ability of MRSA and *Pseudomonas* to interact with *A. castellanii* and epithelial cells. Our findings revealed that MRSA strains exhibited significantly less association with KB epithelial cell line and *Acanthamoeba* strains than *Pseudomonas* strains (Figure 2.2. a and b; Figure 2.3. a and b). Where the association percentage of MRSA with skin epithelial cells was $\sim 0.03\%$, while the association percentage of *Pseudomonas* strains with skin epithelial cell line was $\sim 12.3\%$. Furthermore, MRSA strains were associated with *Acanthamoeba* T1, T4 and T7 with these percentages ~ 0.41 , 0.25 and 0.55% , respectively, while the association percentages of *Pseudomonas* strains with *Acanthamoeba* T1, T4 and T7 were ~ 12.7 , 17 and 16.7% , respectively. *Pseudomonas* 3 exhibited higher association with all *Acanthamoeba* strains (T1, T4 and T7) compared with *P. aeruginosa*₁ and *P. aeruginosa*₂. Interestingly, MRSA₃ exhibited significantly higher association with *Acanthamoeba* T1 and T7 compared with MRSA₁ and MRSA₂ that exhibited similarly percentages of association with all *Acanthamoeba* strains.

Pseudomonas strains exhibited ~ 281 folds higher growth within epithelial cells compared with MRSA, while MRSA strains showed ~ 130 folds higher growth within *Acanthamoeba* compared with epithelial cells.

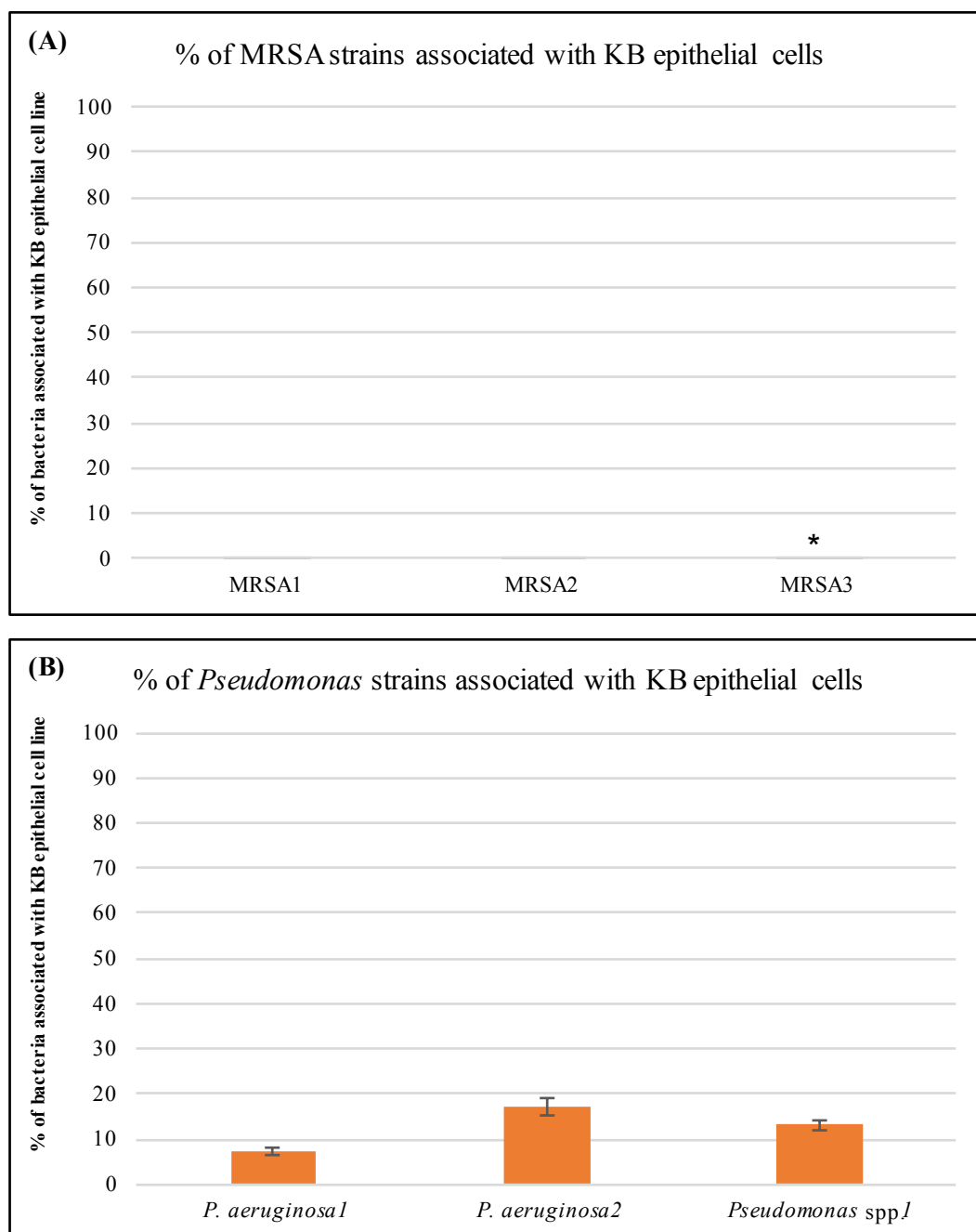


Figure 2.2 Association assays of MRSA and *P. aeruginosa* with KB epithelial cell line.

(A) The percentage of associated MRSA strains with KB epithelial cell line. (B) the percentage of associated *Pseudomonas* strains with epithelial cell line. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

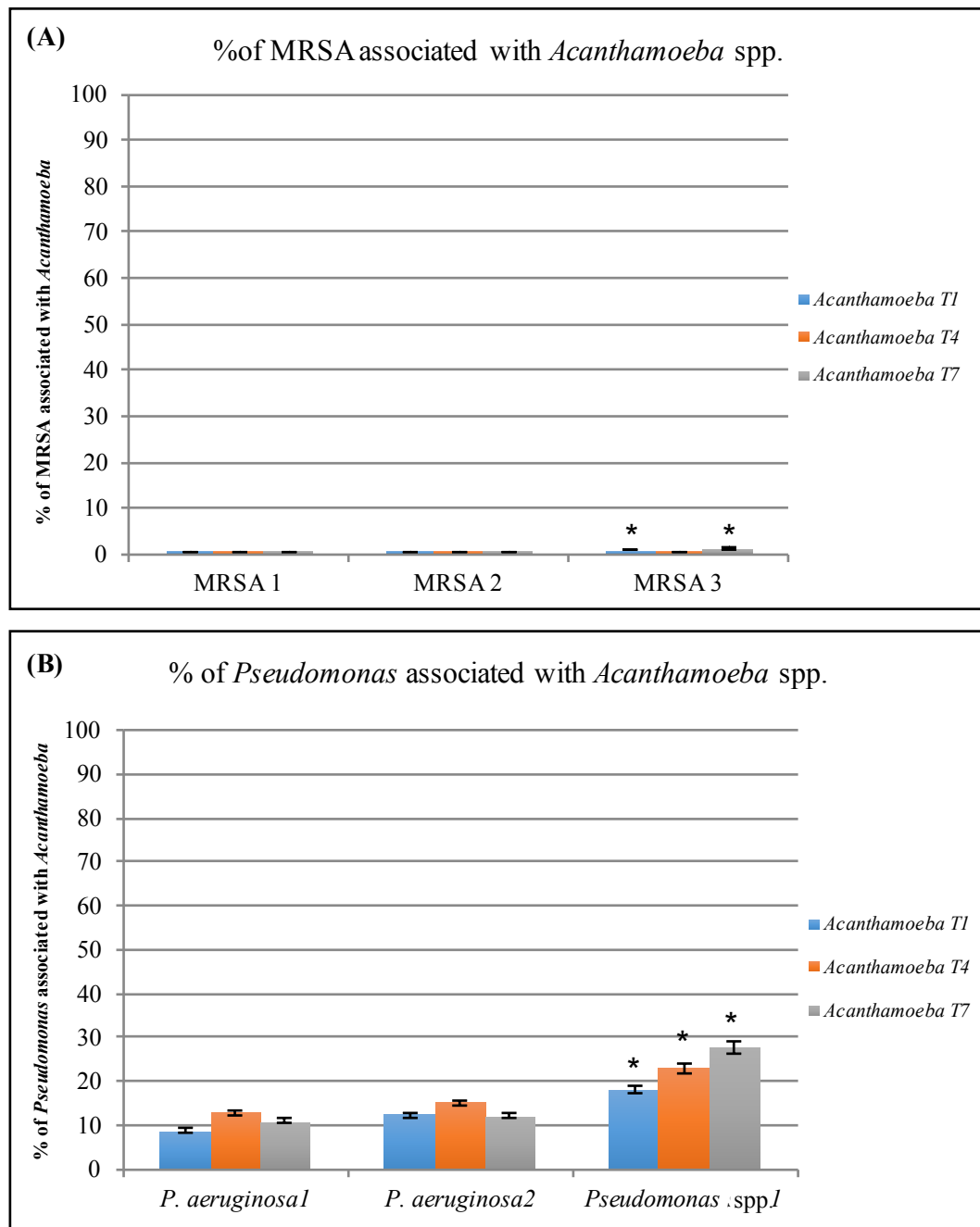


Figure 2.3 Association assays of MRSA and *P. aeruginosa* with *Acanthamoeba* T1, T4 and T7.

(A) The percentage of MRSA strains associated with *Acanthamoeba* strains. (B) the percentage of *Pseudomonas* strains associated with *Acanthamoeba* strains. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

To determine whether *Pseudomonas* and MRSA strains were engulfed by *Acanthamoeba* or KB epithelial cell line, invasion assays were performed. All bacterial strains exhibited recovery from both *Acanthamoeba* strains and epithelial cells (Figure 2.4. a and b; Figure 2.5. a and b). MRSA₃ exhibited significantly higher recovery from epithelial cells compared with MRSA₁ and MRSA₂ as follows: MRSA₃ showed 0.001 % recovery from epithelial cells whereas MRSA₁ and MRSA₂ showed 0.0001 %, respectively 0.0003 % recovery from the epithelial cells. Similarly, MRSA₃ revealed significantly higher recovery with all *Acanthamoeba* strains, particularly T7 as compared with other two strains of MRSA (Figure 2.4. a and Figure 2.5. a).

Interestingly, all *Pseudomonas* strains showed almost similar percentage of recovery from epithelial cells as follows: 0.13 % for *P. aeruginosa*₁, 0.15 % for *P. aeruginosa*₂ and 0.12 % for *Pseudomonas* spp₁. While *Pseudomonas* spp₁ also exhibited higher recovery within all *Acanthamoeba* strains compared to the other two strains of *Pseudomonas*. *Pseudomonas* strains exhibited ~ 333 folds higher growth within epithelial cells compared with MRSA (Figure 2.4. b and Figure 2.5. b).

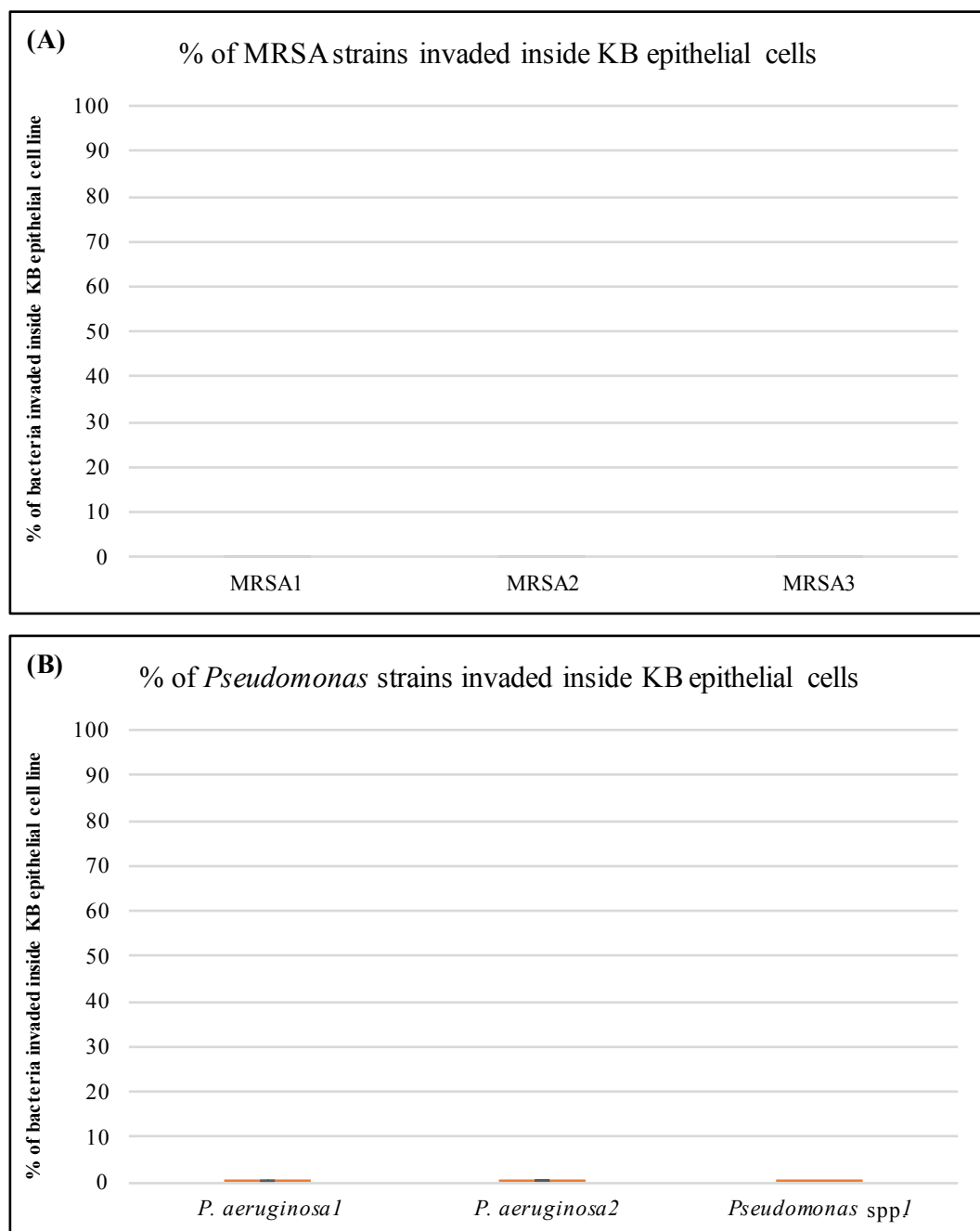


Figure 2.4 Invasion assays of MRSA and *P. aeruginosa* with KB epithelial cell line.

(A) The percentage of MRSA invaded KB epithelial cells, (B) the percentage of *Pseudomonas* strains invaded KB epithelial cells. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

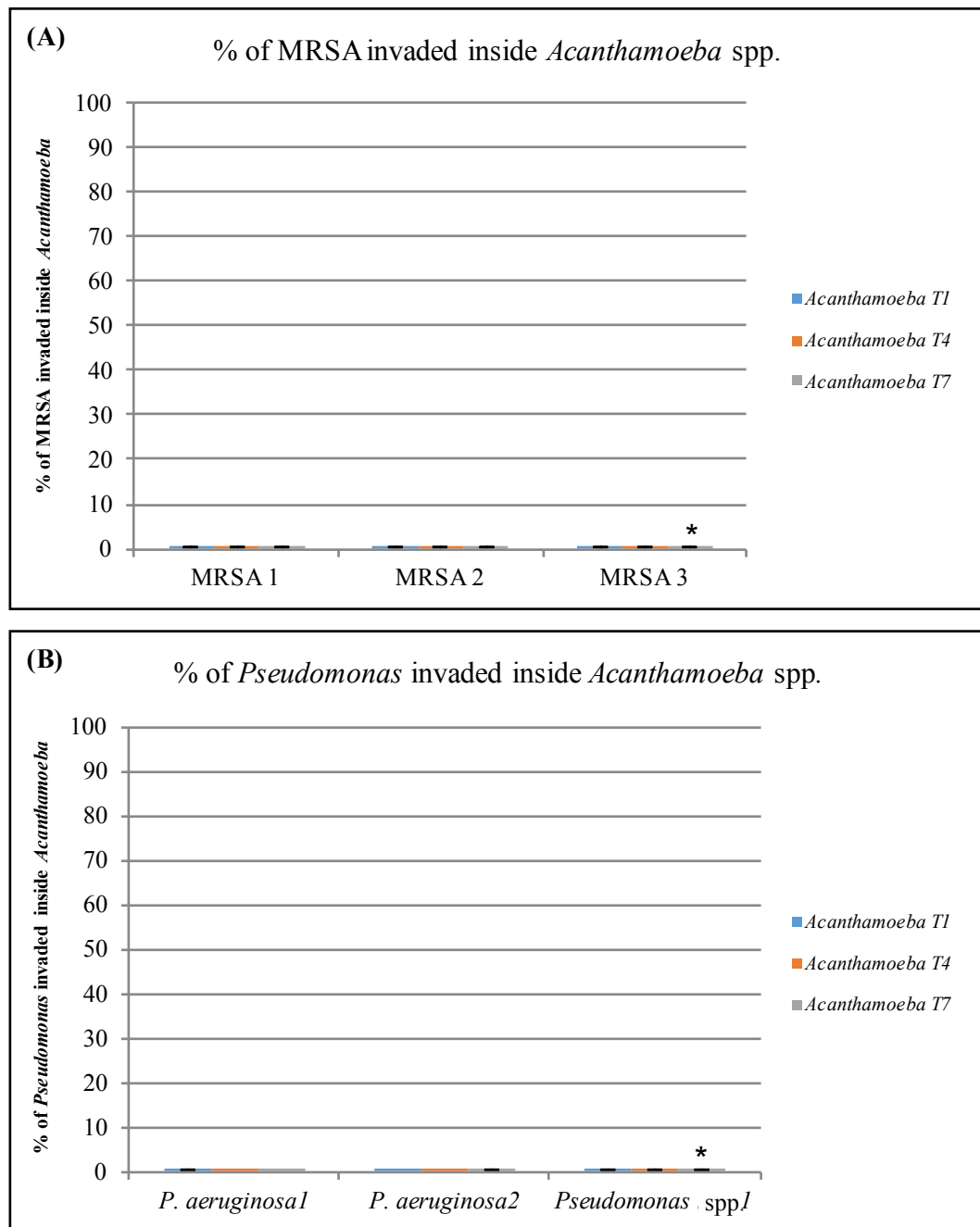


Figure 2.5 Invasion assays of MRSA and *P. aeruginosa* with *Acanthamoeba* T1, T4 and T7.

(A) The percentage of MRSA strains invaded *Acanthamoeba* strains, (B) the percentage of *Pseudomonas* strains invaded *Acanthamoeba* strains. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

To determine the fate of MRSA and *Pseudomonas* survival in long term-interactions with *Acanthamoeba* or skin epithelial cell line (Figure 2.6. a and b; Figure 2.7. a and b), survival assays were performed by incubation of bacteria with *Acanthamoeba* or epithelial cells for 24 h. The findings showed that all MRSA and *Pseudomonas* strains were able to survive and multiply after 24 h of incubation. Interestingly, higher recoveries of MRSA strains were noticed within *Acanthamoeba* strains than their recoveries within epithelial cells. Whereas, *Pseudomonas* strains displayed higher ability to survive inside epithelial cells more than *Acanthamoeba* strains (Figure 2.6. a and b; Figure 2.7. a and b). The survival percentages of MRSA₁, MRSA₂ and MRSA₃ within skin epithelial cell lines were ~ 0.031, 0.017 and 0.044 %, respectively compared with *P. aeruginosa*₁, *P. aeruginosa*₂ and *Pseudomonas* spp₁ recovery after 24 h incubation within skin epithelial cell lines were ~ 13, 15 and 12 %, respectively (Figure 2.6. a and b). However, MRSA and *Pseudomonas* strains were able to survive within *Acanthamoeba* T7 than other strains of *Acanthamoeba*.

Pseudomonas strains exhibited ~ 370 folds higher growth within epithelial cells compared with MRSA while MRSA strains showed ~ 500 folds higher growth with *Acanthamoeba* compared with epithelial cells.

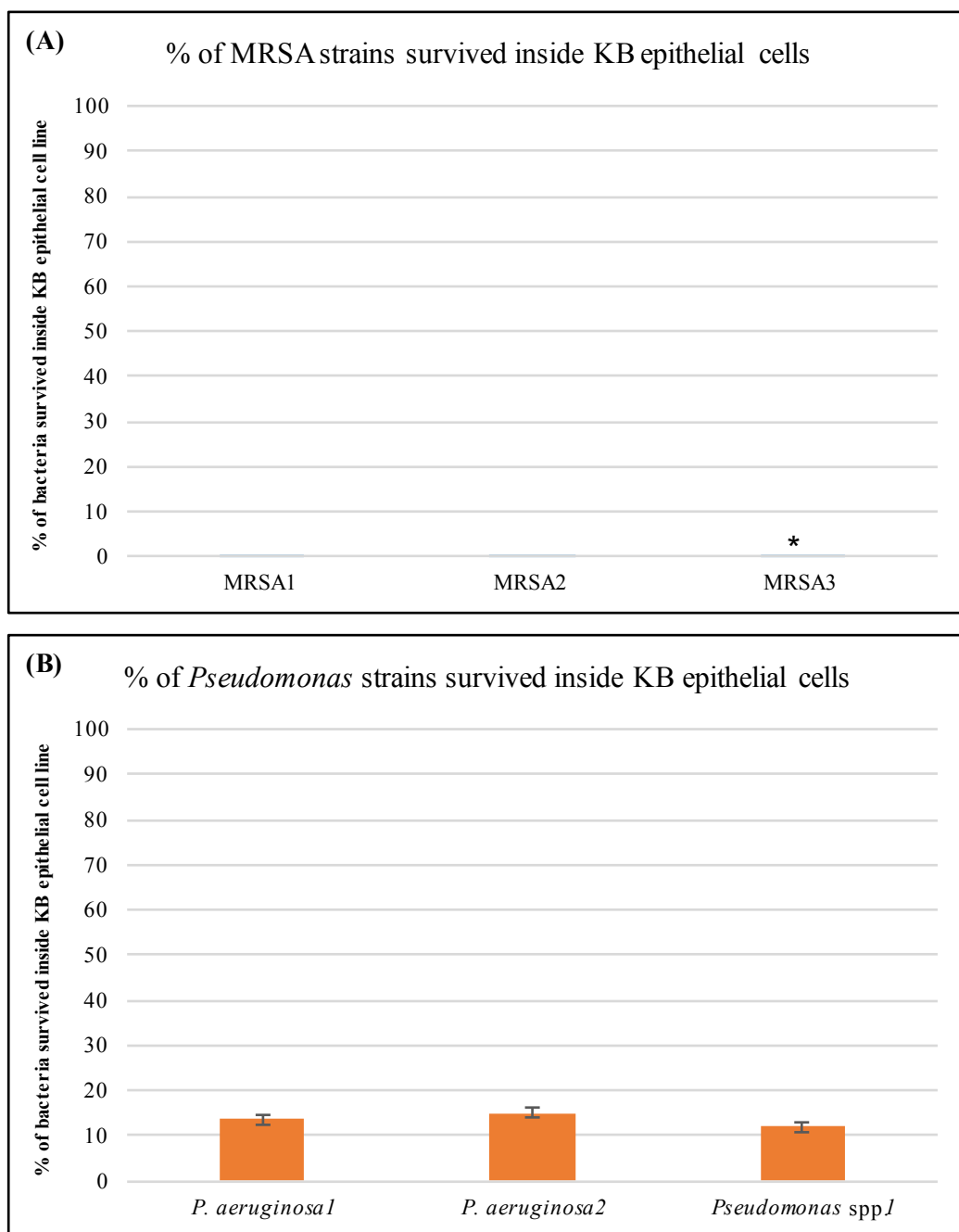


Figure 2.6 Intracellular survival assays of MRSA and *P. aeruginosa* with KB epithelial cell line.

(A) The percentage of MRSA survived inside KB epithelial cell line, (B) the percentage of *Pseudomonas* strains survived inside KB epithelial cell line. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

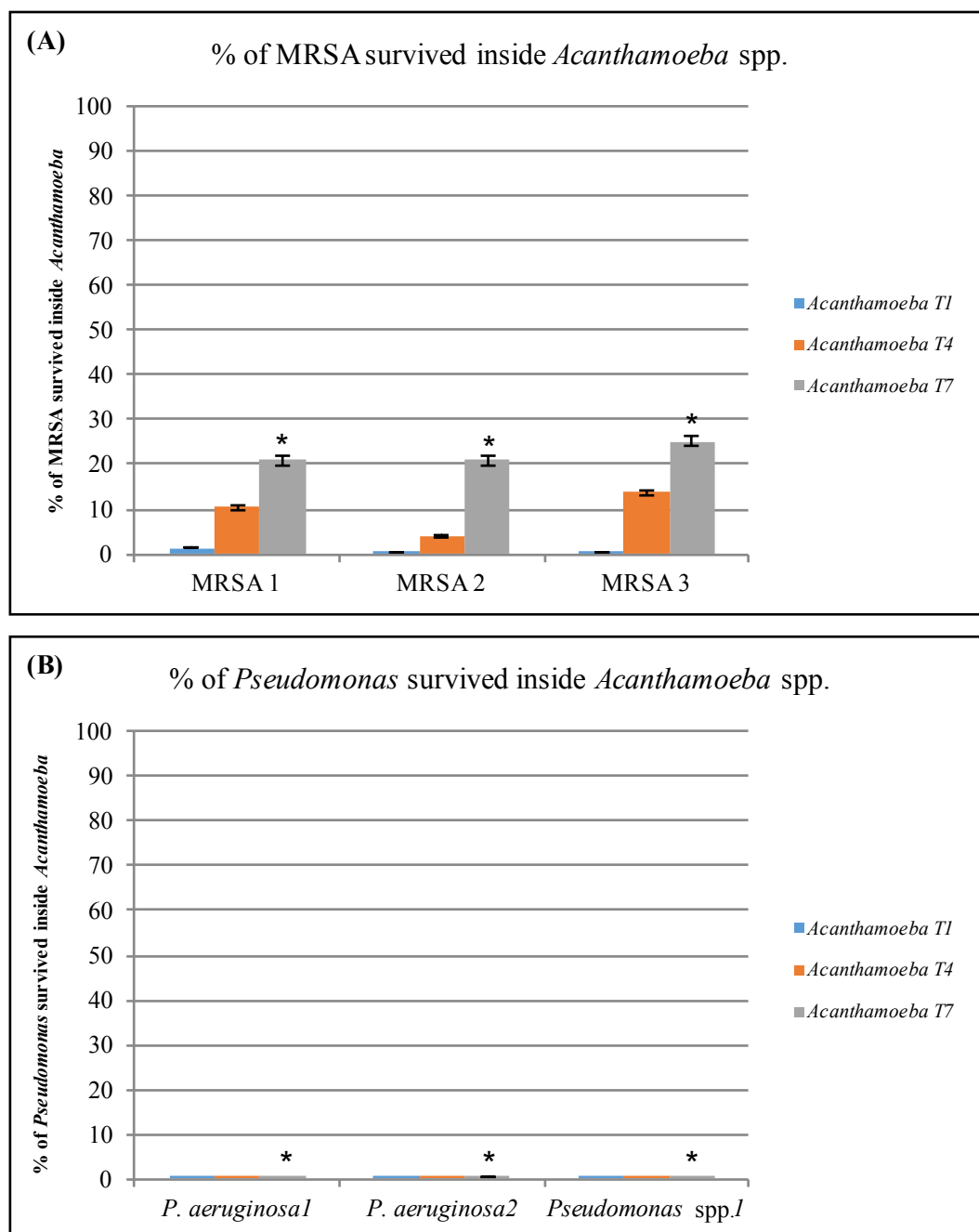


Figure 2.7 Intracellular survival assays of MRSA and *P. aeruginosa* with *Acanthamoeba* T1, T4 and T7.

(A) The percentage of MRSA strains survived inside *Acanthamoeba* strains, (B) the percentage of *Pseudomonas* strains survived inside *Acanthamoeba* strains. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

2.4 Discussion

It is well known that several bacterial pathogens are associated with wound infections with severe consequences; however, the pathogenesis and pathophysiology of these diseases remain unclear. A fundamental question is how do bacteria interact with the epithelial cells, and cause infections. To this end, a skin epithelial cell line was used. Three clinical MRSA strains and three *Pseudomonas* strains isolated from wound infections were used in this study. Furthermore, wounds are one of the common route of *Acanthamoeba* to enter and cause encephalitis, therefore, interaction assays were performed.

In vitro interaction assays showed that, *S. aureus* and *Pseudomonas* have the ability to bind with, invade and survive inside epithelial cells (Ichikawa *et al.*, 2000; Gordon and Lowy, 2008). *S. aureus* has different virulence factors which are responsible for adherence and invasion of epithelial cells, such as protein A, elastin-binding protein, collagen-binding protein, fibronectin-binding protein and clumping factor (Gordon and Lowy, 2008). Pili and LPS in *Pseudomonas* have a role in binding to and invading epithelial cells (Ben *et al.*, 2011). Several lines of evidence have indicated that amoeba serve as a host to a number of human pathogens, including *Mycobacterium avium* (Steinert *et al.*, 1998), *Legionella pneumophila* (Kilvington and Price, 1990), *Escherichia coli* (Alsam *et al.*, 2006), *Enterobacter aerogenes*, *Aeromonas hydrophila* (Yousuf *et al.*, 2013), *P. aeruginosa* (Michel *et al.*, 1995) and MRSA (Cardas *et al.*, 2012) and are able to accommodate bacterial intracellular replication and increase their virulence properties pertinent to susceptible hosts (Abd *et al.*, 2010; Sandström *et al.*, 2010, 2011) Huws *et al.*, (2006) showed MRSA survival and proliferation within *A. polyphaga*. The virulence factors of both bacteria and *Acanthamoeba* may have the role

in the differences between the ability of bacteria to bind with, invade, survive and multiply within host cells.

This is a consequence of the finding that *Pseudomonas* are more likely to bind with and invade *Acanthamoeba* and epithelial cells, and that probably depends on the virulence factors of *Pseudomonas*, such as pili and LPS, which play important roles in adhesion and causing any infections (Hahn, 1997; Ben *et al.*, 2011). However, the ability of *Pseudomonas* to multiply inside *Acanthamoeba* is very low compared with its ability to multiply inside epithelial cells. That would probably rely on the virulence factors of *Acanthamoeba*. *Acanthamoeba* uptake bacteria by using phagocytosis and then use phagolysosomes to lyse them. Although *Acanthamoeba* feed on both Gram-positive and Gram-negative bacteria, they prefer Gram-negative bacteria which are widely used as a food source to isolate *Acanthamoeba* (Khan, 2006). Proteases produced by *Acanthamoeba* are used as significant markers to differentiate between pathogenic and non-pathogenic *Acanthamoeba* species (Khan *et al.*, 2000; Sissons *et al.*, 2006; Dudley *et al.*, 2008). *Acanthamoeba* proteases have antimicrobial effects against different types of bacteria including MRSA and *Pseudomonas* (Iqbal *et al.*, 2014). Our findings show that the environmental, non-pathogenic strain of *Acanthamoeba* (genotype T7), which does not produce any type of proteases, is more likely to host MRSA and *Pseudomonas* than other pathogenic strains of *Acanthamoeba* (genotype T1 and T4). This is interesting as this could be a potential route for bacteria to enter into the human body. Moreover, bacteria can remain and multiply inside *Acanthamoeba* within a harsh environment, and that may help transmit bacterial pathogens to susceptible hosts and also to re-infect wounds again. We demonstrated that the ability of intracellular bacteria to survive inside non-pathogenic *Acanthamoeba* are higher than pathogenic *Acanthamoeba*. In addition, *Pseudomonas* were appropriate food for *Acanthamoeba*. In

conclusion, non-pathogenic *Acanthamoeba* may play role in protecting bacteria during the treatment.

MRSA and *P. aeruginosa* are multidrug resistant; and treatment of infected wounds with these strains are problematic (Gardner *et al.*, 2001). Moreover, *Acanthamoeba* are resistant to many of antimicrobial agents as they protect themselves via encystment process and that would be crucial if there any bacteria inside amoebae (Aksozek *et al.*, 2002). Therefore, the need for alternative active treatment methods is increased. Iron chelators showed antimicrobial activity against range of different bacterial strains (Hider and Kong, 2010), thus, novel iron chelators were used against MRSA, *Pseudomonas* and *Acanthamoeba* strains. These will be discussed in detail in the next chapter (Chapter Three).

Chapter Three

3

Effects of the iron(III)- selective hexadentate-ligands of iron chelators as antimicrobial agents against MRSA and *Pseudomonas* strains and *Acanthamoeba* spp.

Chapter 3 : Effects of the iron(III)- selective hexadentate-ligands of iron chelators as antimicrobial agents against MRSA and *Pseudomonas* strains and *Acanthamoeba* spp.

3.1 Introduction

Nosocomial infections caused by antibiotic-resistant bacteria are becoming more common (Hogan and Kolter, 2002). The severity of these infections is variable, between superficial only, such as cellulitis, and severe infection, such as Staphylococcal Scalded Skin Syndrome (SSSS) (Plowman, 2000). The healthcare associated infections (HAI) are amongst the important factors which would lead to prolonged stays in hospital, therefore increasing the cost of wound care (Talan *et al.*, 2011). It is a big task nowadays for clinicians to manage and keep wound infections under control. Wound infections involve a complex interaction between host, pathogens and the environment. However, the molecular mechanisms of the interaction of host and pathogen are crucial and not completely understood (Cooper, 2005). Both MRSA and *P. aeruginosa* are multi-drug resistant bacteria because of the over use and misuse of antibiotics. MRSA has been directly associated with severe wound infections and has resulted in an increase in morbidity and mortality rates (Gardner *et al.*, 2001). Treatment is problematic, because of their increased virulence factors, such as penicillin-binding protein 2A (PBP2A) in MRSA that is responsible for antibiotic resistance. Therefore, the need for alternative treatment methods has become very important.

Iron is an essential cofactor of many biochemical pathways in both prokaryotic and eukaryotic species (Thompson *et al.*, 2012). Hence, decreasing the availability of iron should, in principle, inhibit microbial growth (Lewin, 1984). There are many strategies used by many different microorganisms to absorb and scavenge iron from their environment, such as producing and secreting siderophores, which have a high affinity

and selectivity for iron(III) (Hider and Kong, 2010). *S. aureus* use two types of carboxylate family siderophores, which are staphyloferrin A and staphyloferrin B, via the transporters Hts and Sir, respectively, to access the transferrin iron pool (Beasley *et al.*, 2011). *Pseudomonas aeruginosa* produces two types of siderophores, which are pyoverdine and pyochelin. Moreover, *P. aeruginosa* uses a large number of xenosiderophores to uptake the iron molecule by a mechanism known as siderophore piracy (Youard *et al.*, 2011). The presence of the high-affinity iron-selective chelating agents can interrupt the iron uptake (Bergan *et al.*, 2001). The affinity of these agents must be extremely high to be able to efficiently out-compete the native siderophores for iron. Therefore, siderophores would not be able to scavenge iron molecules when the high affinity hexadentate hydroxypyridinone binds with iron (Hider and Kong, 2010). The design of iron chelators for an antimicrobial purpose should not have one of the structures which bind with siderophores, including hydroxamate, catecholate and α -hydroxycarboxylate, otherwise the iron chelator's complex may well be able to supply iron to the microorganism via the iron-siderophore transporter (Ji *et al.*, 2012). Three recent studies have shown the antimicrobial effect of hexadentate 3-hydroxypyridin-4-ones (HPO) on both Gram-positive and Gram-negative bacteria (Qiu *et al.*, 2011; Xu *et al.*, 2011; Ma *et al.*, 2014). All compounds which have been developed and used in those studies have a different structure from all other known siderophores; therefore, the iron complex cannot gain access to the bacteria by receptor mediated transport.

Herein the antimicrobial activity of HPO with high affinity for iron (III) against MRSA and *P. aeruginosa* isolates alone, against *Acanthamoeba* strains (T4 and T7), and the intracellular bacterial strains inside *Acanthamoeba* and KB epithelial cell line are described. Furthermore, the cytotoxic effect of bacterial strains in the presence and

absence of HPO, and whether these chelators would stop or reduce *Acanthamoeba* proteases expression were tested.

3.2 Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

3.2.1 Cell lines and cell culture

KB epithelial cell line was grown and culture as described in section 2.2.1.

3.2.2 *Acanthamoeba* culture

Acanthamoeba strains (T4 and T7 genotypes) were grown and cultured as described in section 2.2.2.

3.2.3 Bacterial culture

MRSA and *Pseudomonas* strains were cultured, inoculated in LB broth and OD were measured by spectrophotometer as described in section 2.2.3.

3.2.4 Preparation of antimicrobial agents

Three novel iron chelators (13a, 13l, and 13i) were designed and synthesised for this study by Dr Tao Zhou from Zhejinag Gongshang University, China. The iron chelator 3-Hydroxy-1,2-dimethyl-4(*IH*)-pyridone (deferiprone) was used as a control. All compounds were tested in five concentrations for their antimicrobial effects against MRSA and *Pseudomonas* strains. The solutions of these compounds were prepared by dissolving the chelator in destil sterile water (dsH₂O).

The chemical structures of compounds are shown in Figure 3.1.

Chemical structure of the four iron chelators

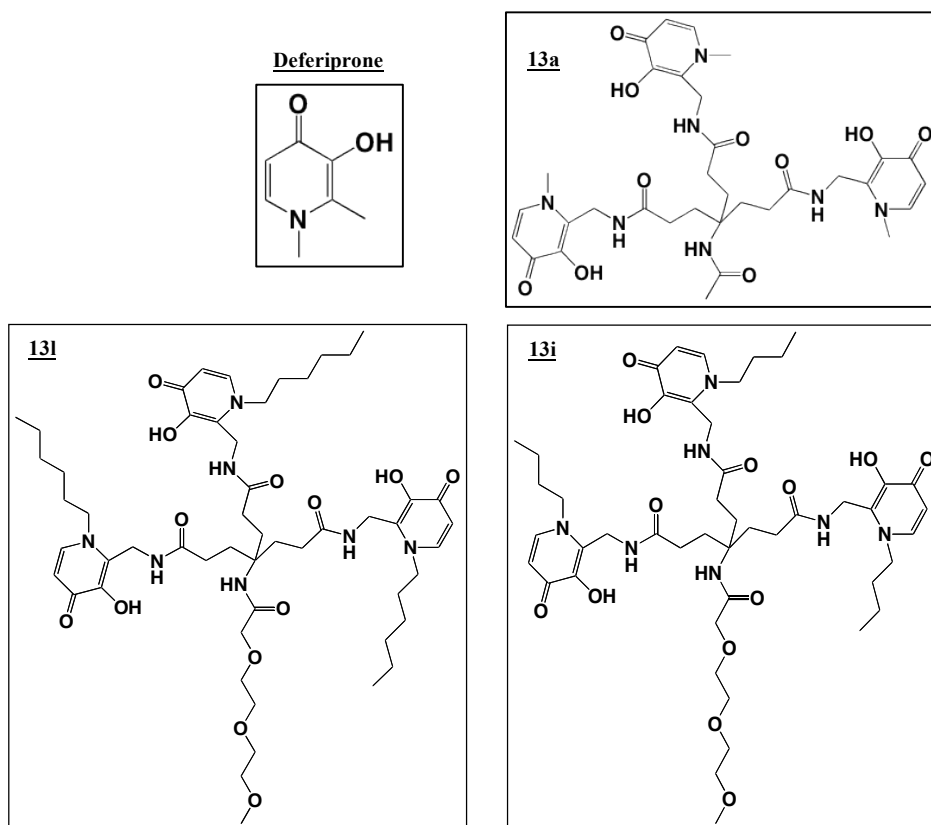


Figure 3.1 Chemical structure of deferiprone and the novel iron chelators.

Figure adapted from (Zhou *et al.*, 2015).

3.2.5 Antimicrobial assays

3.2.5.1 Antibacterial assay

To determine the antimicrobial activity of the iron (III)- selective HPO chelators to inhibit the bacterial growth, antimicrobial assays were performed. Bacterial strains were grown in LB broth overnight at 37 °C. Then ODs were measured by spectrophotometer as described above. After measuring, each strain was diluted (1:1) in dsH₂O to give final number of bacteria ($\sim 0.5 \times 10^8$ CFU/mL). Five different final concentrations of iron chelators (100, 200, 300, 400 and 500 µg/mL) were used in this assay. 96-well plates were used to perform this assay. All wells contained 80 µL of antimicrobial agent in sterilized water and 20 µL of bacteria ($\sim 1 \times 10^7$ CFU/well), except wells of controls that contained 80 µL of sterilized water with 20 µL ($\sim 1 \times 10^7$ CFU/well) of bacteria. Then, bacteria with iron chelators were incubated at 37 °C for 6 and 24 h. The number of viable bacteria in each well was determined by plating onto CLED agar using 20 µL volumes in duplicate. The bactericidal rate was calculated as follows:

$$\left(R = \frac{[X_0 - X_t]}{X_0} \times 100 \right)$$

where R is the bactericidal rate, X₀ the number of bacteria without chelator (control), and X_t the number of bacteria after the treatment with chelator (Qiu *et al.*, 2011).

3.2.5.2 Antiamoebic assays

To determine the antimicrobial activity of the iron (III)- selective HPO chelators on *Acanthamoeba*, antiamoebic assays were performed. In brief, *Acanthamoeba* were cultured and grown in 96-well plates in PYG ($\sim 1 \times 10^5$ cells per 0.150 mL in each well); next, plates were incubated until confluent. Then, the growth medium was discarded and wells washed with PBS once to remove floating *Acanthamoeba*. Five different final concentrations of iron chelators (100, 200, 300, 400 and 500 µg/mL) were added to

different wells. All wells contained 150 μL of antimicrobial agent in dsH_2O , except wells of controls that contained only 150 μL of sterilised water. Then *Acanthamoeba* with iron chelators were incubated at 30 °C for 6 and 24 h. The number of viable and unviable *Acanthamoeba* in each well was determined by adding 150 μL of 0.4 % Trypan blue in each well and incubating for 15 min. The numbers of viable and unviable *Acanthamoeba*, as judged by dye exclusion or staining, were counted by a haemocytometer.

3.2.5.2.1 Conditioned medium (CM)

To determine the effects of iron chelators on the extracellular proteases released from intact *Acanthamoeba*, CM was produced by inoculating *Acanthamoeba* with and without iron chelators in serum free medium (RPMI) for 24 h. Next day, CM was collected by centrifugation and used for zymography (Alsam *et al.*, 2005b; Dudley *et al.*, 2008).

3.2.5.2.2 Zymography

Zymographic assays were performed to visualise and begin to characterise *Acanthamoeba* extracellular proteases. Briefly, CM was mixed (1:1) with sample buffer (containing 4 % sodium dodecyl sulfate (SDS)) and electrophoresed on SDS-polyacrylamide gels (SDS-PAGE) containing gelatin (1 mg/mL). After electrophoresis, gels were soaked in 2.5 % Triton X-100 (w/v) for 60 min to remove the SDS. Finally, the gels were incubated in a developing buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM CaCl_2) at 37 °C overnight, rinsed, and stained with Coomassie brilliant blue. Areas of gelatin digestion indicating protease activities are seen as non-staining regions in the gel (Alsam *et al.*, 2005b; Dudley *et al.*, 2008).

3.2.6 Intracellular antimicrobial assays

3.2.6.1 *Acanthamoeba* intracellular uptake

In brief, *Acanthamoeba* were cultured in 96-well plates in PYG (~1x10⁵ cells per 0.150 mL in each well); and plates were incubated until confluency. Then, the growth medium was discarded and wells washed with PBS once to remove floating *Acanthamoeba*. Next, bacterial strains (~1.2x10⁶ CFU/well) were added onto *Acanthamoeba* with RPMI medium and incubated for 1 h at room temperature. Then, supernatants were discarded and wells washed by PBS once to remove non-adherent bacteria. Next, the wells were washed once again with PBS, and then antibiotics were added to kill extracellular bacteria (vancomycin against MRSA, and ciprofloxacin against *Pseudomonas*; 100 µg/mL final concentration, for 45 min). Then the supernatants were discarded and wells washed once with PBS. The final five different concentrations of iron chelators (100, 200, 300, 400 and 500 µg/mL) were used in this assay. All wells contained 150 µL of antimicrobial agent in sterilized water, except wells of controls that contained only 150 µL of sterilized water. Then, *Acanthamoeba* with bacteria inside and iron chelators were incubated at 30 °C for 6 and 24 h. Next, *Acanthamoeba* were lysed by adding 0.5 %SDS final concentration for 10 min. Finally, 20 µL of lysed solution was cultured onto CLED agar, and incubated overnight at 37 °C in air incubator. The bactericidal rates of iron chelators on intracellular bacteria within *Acanthamoeba* were calculated as follows:

$$\left(R = \frac{[X_0 - X_t]}{X_0} \times 100 \right)$$

Where R is the bactericidal rate, X₀ is the number of intracellular bacteria without chelator (control), and X_t represent the number of intracellular bacteria after the treatment with chelator.

3.2.6.2 *KB epithelial intracellular uptake*

To investigate the effects of the iron (III)- selective HPO chelators on the intracellular bacteria within KB epithelial cell line, antimicrobial assays were performed. In brief, KB epithelial cells were cultured and grown in 96-well plates in EMEM ($\sim 1 \times 10^5$ cells per 0.150 mL in each well); then plates were incubated until confluency. Then, the growth medium was discarded and wells washed with PBS once to remove floating cell. Next, bacterial strains ($\sim 1.2 \times 10^6$ CFU/well) were added onto cells with EMEM medium and incubated for 1 h in CO₂ Incubator (37 °C in humidified 95 % air, 5 % CO₂). Then, supernatants were discarded and cells were washed with PBS once to remove non-adherent bacteria. Next, antibiotics were added to kill extracellular bacteria (vancomycin against MRSA, and ciprofloxacin against *Pseudomonas*; 100 µg/mL final concentration, for 45 min). Then the supernatants were discarded and wells washed once with PBS. Following this, five different concentrations of iron chelators (as mentioned above in section 3.2.6.1.) were used in this assay. All wells contained 150 µL of antimicrobial agent in EMEM medium, except wells of controls that contained only 150 µL of EMEM medium. The KB epithelial cell line with bacteria inside and iron chelators were incubated in CO₂ incubator (37 °C in humidified 95 % and, 5 % CO₂) for 6 and 24 h. Then, KB epithelial cells were lysed by adding 150 µL of CellLytic M[®] for 10 min. In the end, 20 µL of lysed solution was cultured onto CLED agar, and incubated for overnight at 37 °C in air incubator.

3.2.7 Cytotoxicity assays

This assay was performed to determine the cytotoxic effect of MRSA and *Pseudomonas* on KB epithelial cell line. In brief, the method used here is as previously described in section (2.6.). After the incubation time, the supernatants were collected in centrifuge tubes to determine the cytotoxic effect of these bacteria on KB cells. In addition, the

cytotoxic effect was assessed in three methods. The first method was by staining the cells with hematoxylin stain and assessing visually. The second method was by staining with Trypan blue and by counting viable and dead cells, which will be mentioned in the next section. The third method was by measuring Lactate DeHydrogenase (LDH) by using a specific kit (Alsam *et al.*, 2003).

3.2.7.1 Cell visualising

Cytotoxic effects were assessed visually by hematoxylin staining. Briefly, the supernatants were collected to use for the LDH assay in the next section (3.2.7.2.); then cells were fixed by acetone and methanol 1:1 for 15 min. Then, hematoxylin stain was added to the cells to stain for 30 min. In the end, the stained cells were assessed visually under the microscope (Alsam *et al.*, 2003).

3.2.7.2 Measurements of lactate dehydrogenase (LDH)

LDH is an enzyme that is released when cells damaged. Cytotoxic effects were determined by measuring LDH release into the supernatants (Cytotoxicity detection kit^{plus}; Roche). Briefly, the supernatants which had been collected, were centrifuged (10000 x g for 5 min). Next, 50 µl of supernatants were loaded in 96-well plates. Moreover, high and low controls were prepared as follows: lysis buffer was added onto the control cells with growth medium to release a high amount of LDH, while the low control was just control cells with growth medium. Then 50 µl of reaction mixture were added to each sample and incubated for 15 min at room temperature in the dark. Next, the stop solution was added to stop the reactions (Alsam *et al.*, 2003). Finally, the 96-well plates were shaken for 10 seconds and read by multiplate reader [FLUOstar Omega] at wavelength 600. The percentage cytotoxicity was calculated as follows:

$$\left(\% \text{ Cytotoxicity} = \frac{[\text{Sample value} - \text{Low control value}]}{[\text{High control value} - \text{Low control value}]} \times 100 \right)$$

3.2.8 Software and statistics:

All graphs and data analysis were done as describe in section (2.2.7.). Microsoft Office PowerPoint was used to adjust the contrast and brightness of photos.

3.3 Results

3.3.1 Iron chelators as antimicrobial agents

The viable bacteria were determined after incubation of bacteria for 6 and 24 h in the presence or absence of the novel iron chelators (13a, 13i and 13l) compared with deferiprone. 13l was found to exhibit the strongest inhibitory activity against MRSA₁ amongst the tested chelators after both 6 and 24 h incubation (Figure 3.2.). At 6 h incubation, hexadentate chelators 13a and 13i showed stronger inhibition on MRSA₁ than deferiprone (Figure 3.2.). Compared with deferiprone, 13a exhibited similar inhibitory activity after 24 h incubation, while 13i was much weaker. In the cases of MRSA₂ and MRSA₃, similar results were found, chelator 13l exhibiting the strongest inhibitory activity throughout. After 24 h incubation, the inhibition rates of 13l at a concentration of 400 mg/mL against MRSA₁, MRSA₂ and MRSA₃ were determined to be 96.8 %, 95.6 % and 97.2 %, respectively, while the inhibition rates of the control chelator (deferiprone) at the same concentration against MRSA₁, MRSA₂ and MRSA₃ were determined as 80.1 %, 83.1 % and 87.6 %, respectively (Table 3.1). Overall, the *Pseudomonas* strains were found to be more sensitive to the iron chelators than the MRSA strains. Surprisingly, at concentrations less than 200 µg/mL, all three compounds (13a, 13i and 13l) failed to exhibit superior inhibitory activity against the three strains of *Pseudomonas* when compared with deferiprone (Figure 3.3.). However, at higher concentrations, these three compounds were found to exhibit a stronger inhibitory effect compared with deferiprone. After incubation for 24 h, all three strains of *Pseudomonas* were killed when the concentration of hexadentate chelator (13a, 13i and 13l) was over 400 µg/mL, while the bacterial inhibition rates of deferiprone at a concentration of 400 µg/mL against *P. aeruginosa*₁, *P. aeruginosa*₂ and *Pseudomonas* spp₁ were determined to be 93.1 %, 92.3 % and 92.1 %, respectively (Table 3.2).

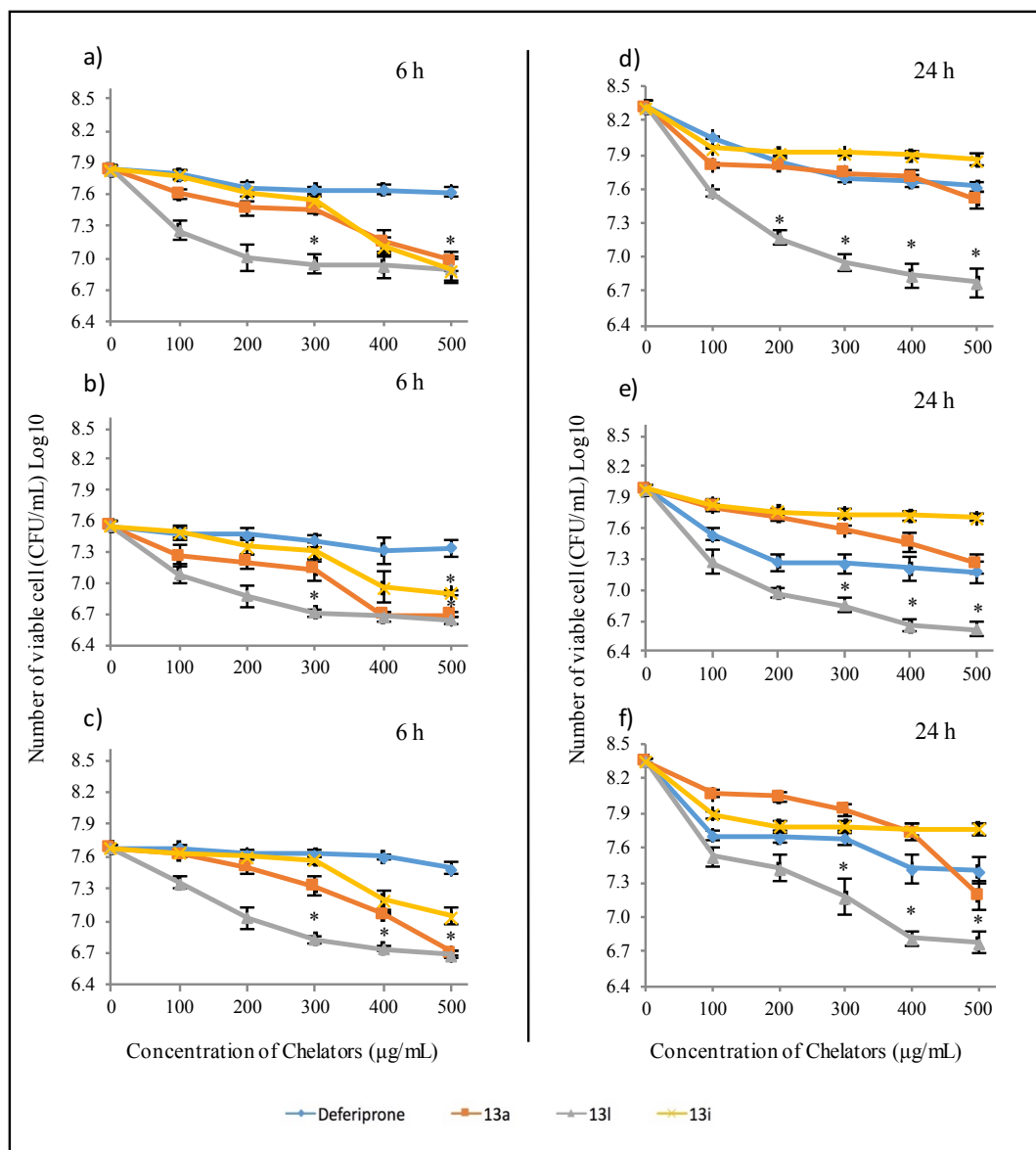


Figure 3.2 The growth inhibition of MRSA strains after treatment with different concentrations of novel iron chelators compared with deferiprone.

(a, b and c) growth of MRSA₁, MRSA₂ and MRSA₃, respectively, after 6 h incubation. (d, e and f) growth of MRSA₁, MRSA₂ and MRSA₃, respectively, after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = P<0.05).

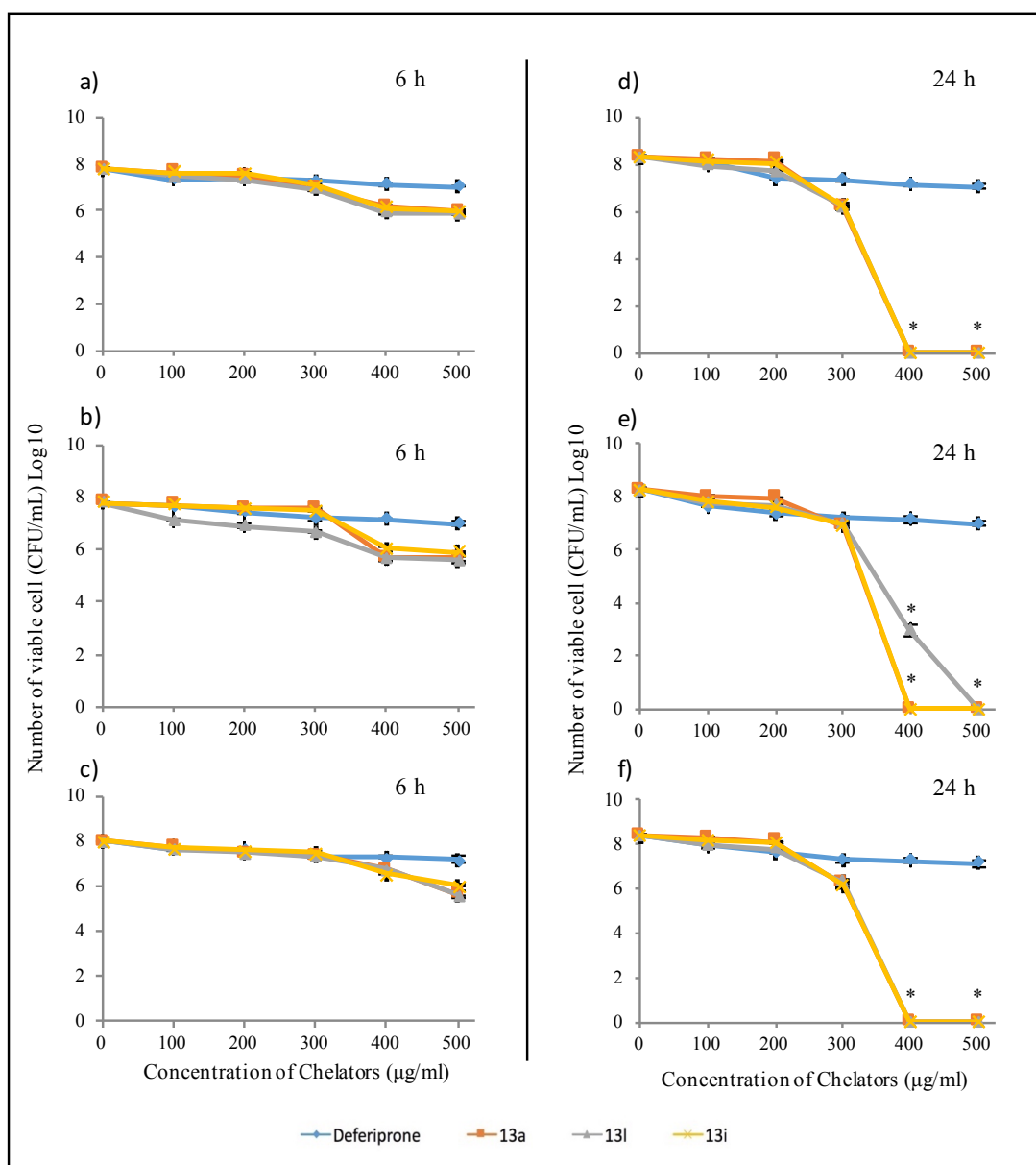


Figure 3.3 The growth inhibition for *Pseudomonas* strains after treatment with different concentrations of novel iron chelators compared with deferiprone.

(a, b, and c) growth of *P. aeruginosa*₁, *P. aeruginosa*₂ and *Pseudomonas* spp₁, respectively, after 6 h incubation. (d, e, and f) growth of *P. aeruginosa*₁, *P. aeruginosa*₂ and *Pseudomonas* spp₁, respectively, after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

	MRSA ₁		MRSA ₂		MRSA ₃	
	6 h	24 h	6 h	24 h	6 h	24 h
Deferiprone (3.60 mM)	38.8	80.1	37.4	83.1	33.2	87.6
13a (0.62 mM)	85.5	84.4	86.6	80.0	89.4	92.1
13l (0.44 mM)	87.3	96.8	88.1	95.6	89.7	97.2
13i (0.48 mM)	88.0	56.6	78.2	46.9	75.8	74.0

Table 3.1 Bacterial inhibition rate (%) of iron chelators on MRSA strains after 6 and 24 h incubation at concentration of 500 µg/mL.

	<i>P. aeruginosa</i> ₁		<i>P. aeruginosa</i> ₂		<i>Pseudomonas</i> spp. ₁	
	6 h	24 h	6 h	24 h	6 h	24 h
Deferiprone (2.88 mM)	78.1	93.1	75.4	92.3	78.7	92.1
13a (0.50 mM)	97.5	100	99.2	100	94.1	100
13l (0.35 mM)	98.7	100	99.2	100	94.2	100
13i (0.38 mM)	97.8	100	98.2	100	96.1	100

Table 3.2 Bacterial inhibition rate (%) of iron chelators on *Pseudomonas* strains after 6 and 24 h incubation at concentration of 400 µg/mL.

3.3.2 Iron chelators as antiamoebic agents

Antiamoebic assays were performed to determine whether the iron chelators such as 13a, 13l, 13i and deferiprone have amoebicidal activities against *Acanthamoeba* T4 and T7 in trophozoite stage. The results showed that after 6 h incubation, 13a at 100 µg/mL had 100 % amoebicidal (trophozoites were killed) activity against both strains of *Acanthamoeba* [T4 and T7], while 13l and 13i had 100 % at 300 and 400 µg/mL, respectively, compared with deferiprone which did not show a significant activity against both genotypes (Figure 3.4.). As shown in (Figure 3.4.), 13l decreased the number of viable *Acanthamoeba* T4 trophozoites at 100 µg/mL after 6 h incubation from 18×10^4 trophozoite/mL to 9×10^4 trophozoite/mL with a amoebicidal rate 52 %, while 13i at the same concentration after 6 h number of viable *Acanthamoeba* T4 trophozoites decreased to be 11×10^4 trophozoites/mL with a amoebicidal rate 3 %. However, deferiprone at 500 µg/mL did not have a real effect on *Acanthamoeba* T4 trophozoites after 6 and 24 h, whereas, this chelator decreased the viable trophozoites from 18×10^4 trophozoite/mL to be about 16×10^4 trophozoites/mL and 13×10^4 trophozoite/mL, respectively, with amoebicidal rates 13 and 20 %. Moreover, 100 µg/mL of 13l and 13i chelators decreased the number of viable *Acanthamoeba* T7 trophozoites after 6 h incubation from 24×10^4 trophozoite/mL to 15×10^4 trophozoites/mL and 24×10^4 trophozoite/mL, with amoebicidal rate 36 and 12 %, respectively. Furthermore, 200 µg/mL of same chelators after 6 h incubation time decreased the number of viable cells from 24×10^4 trophozoite/mL to 9×10^4 trophozoite/mL and 16×10^4 trophozoite/mL, respectively, with amoebicidal rates 61 and 32 % respectively. As shown in (Figure 3.5.), all novel iron chelators at 500 µg/mL had antiamoebic activity on *Acanthamoeba* trophozoites compared with deferiprone which did not show any activity at the same concentration.

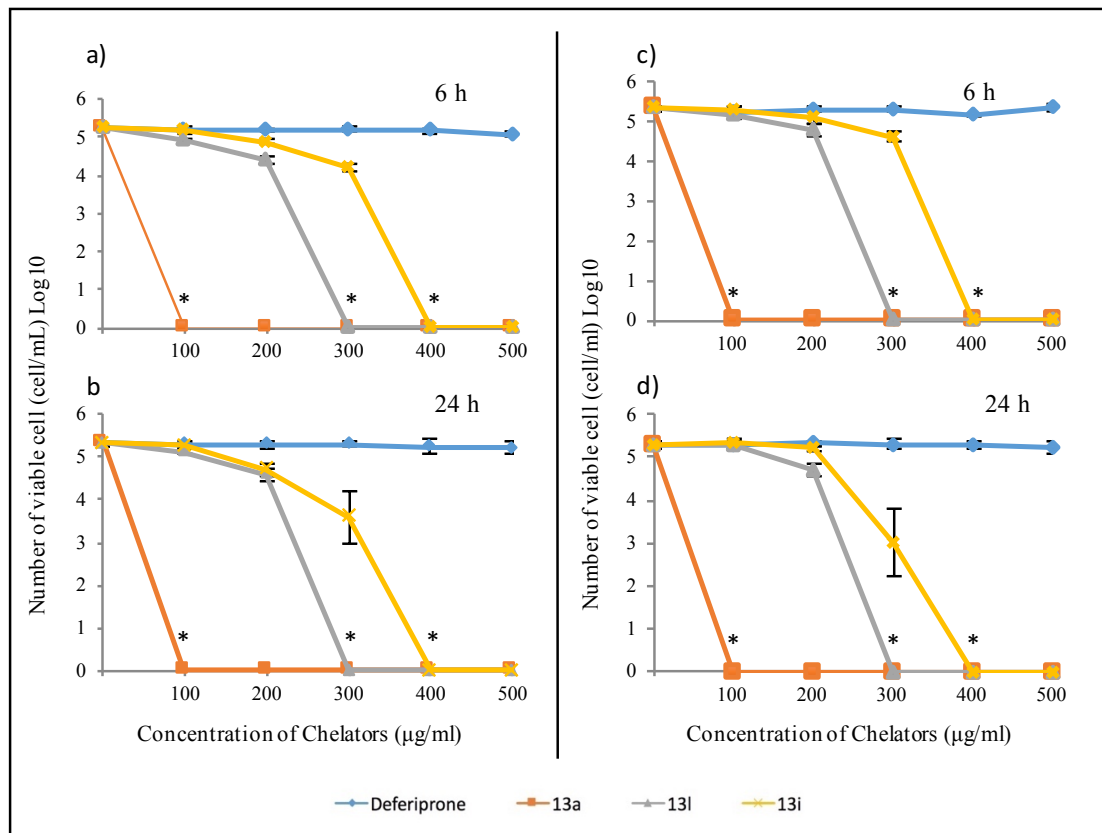


Figure 3.4 The antiamoebic effect of iron chelators on *Acanthamoeba* (T4 and T7) trophozoites after treatment with different concentrations of iron chelators.

(a and b) Number of viable *Acanthamoeba* T4 after 6 and 24 h incubation, respectively. (c and d) Number of viable *Acanthamoeba* T7 after 6 and 24 h incubation, respectively. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

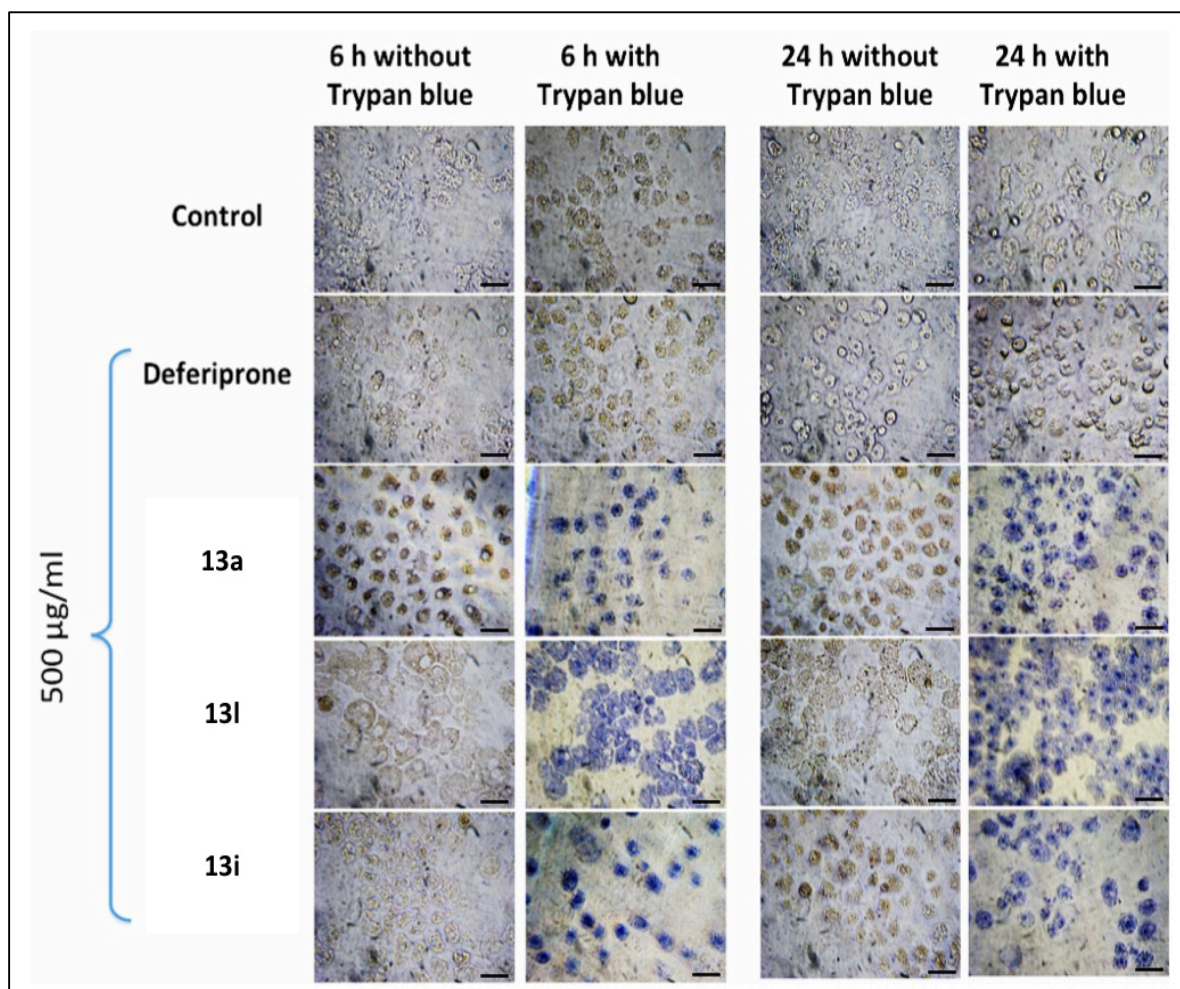


Figure 3.5 Trypan blue viability test.

Trypan blue viability test of *Acanthamoeba* T4 trophozoite after treated with 500 µg/mL of the novel iron chelators compared with deferiprone. (*Acanthamoeba* trophozoites, bars = 50 µm).

3.3.3 Effect of iron chelators on the intracellular bacteria inside *Acanthamoeba*

Antimicrobial assays were performed to determine whether the iron(III)- selective hexadentate ligands of 3-hydroxypyridine-4-one chelators have bactericidal activities against MRSA and *Pseudomonas* within pathogenic and non-pathogenic *Acanthamoeba* trophozoites. Our findings revealed that all iron chelators almost inhibit the growth of intracellular MRSA at the lowest concentration (100 µg/mL) for 6 h. However, after 24 h incubation the number of remaining viable MRSA multiplied. All novel iron chelators including 13a, 13l and 13i and the control chelator had antimicrobial activity after 6 h incubation against intracellular MRSA within *Acanthamoeba* T4 trophozoites at 100, 300, 300 and 300 µg/mL, respectively. Concentrations of 13a, 13l, and 13i (100, 200, and 200 µg/mL, respectively), were sufficient to eliminate both *Acanthamoeba* T7 and intracellular MRSA. In order to determine whether any bacteria remained inside *Acanthamoeba* which could multiply significantly, the experiments were repeated and 96-well plates incubated for 24 h. The Minimum Bactericidal Concentration (MBC) of 13a, 13l and 13i after 24 h incubation were 100, 500 and 500 µg/mL, respectively, against MRSA inside *Acanthamoeba* T4, compared with deferiprone that showed viable MRSA at 500 µg/mL. While the MBC values for them against MRSA inside *Acanthamoeba* T7 were 100, 300 and 500 µg/mL, respectively, with bactericidal rate of 100 % for all; by comparison deferiprone did not show activity against intracellular MRSA at 500 µg/mL (Figure 3.6.).

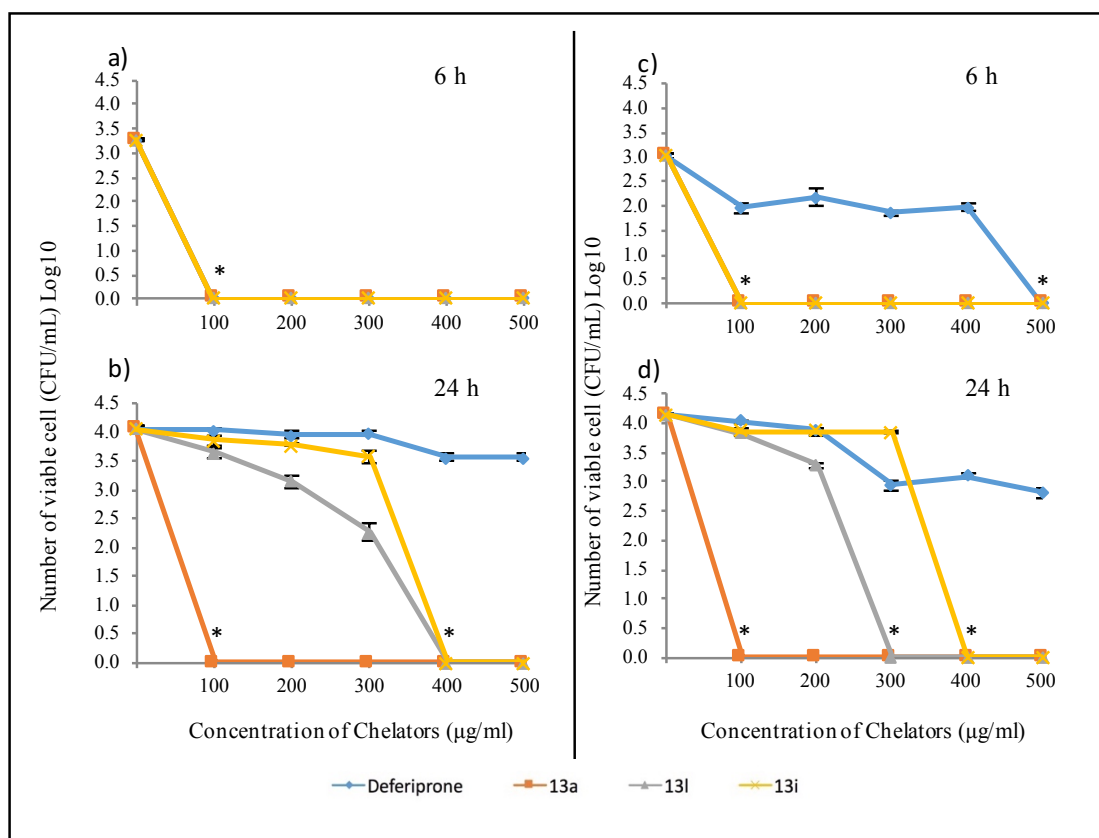


Figure 3.6 The antimicrobial effect of iron chelators on intracellular MRSA inside *Acanthamoeba* T4 and T7 trophozoites after treatment with different concentrations of iron chelators.

(a and b) Number of recovered MRSA from within *Acanthamoeba* T4 after 6 and 24 h incubation, respectively. (c and d) Number of recovered MRSA from within *Acanthamoeba* T7 after 6 and 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

The inhibitory effect of the chelators against the growth of intracellular *P. aeruginosa* is illustrated in (Figure 3.7.). As shown previously in this chapter, 13a had the highest effect on *Acanthamoeba* trophozoites and bacteria within. All the novel iron chelators showed antimicrobial activity compared with deferiprone which had no significant effect against intracellular *P. aeruginosa*. The lowest concentration (100 µg/mL) of 13a completely eliminated intracellular *P. aeruginosa*, with bactericidal rate 100 %. The MBC values of 13l and 13i after 6 h incubation against *P. aeruginosa* inside the trophozoite of *Acanthamoeba* T4 were 300 and 400 µg/mL, respectively, while the MBC for both chelators were 400 µg/mL against *P. aeruginosa* inside the T7 trophozoite. The results after 24 h incubation showed that 13l and 13i had antimicrobial effect against *P. aeruginosa* inside both T4 and T7 trophozoite, whereas 400 and 500 µg/mL of 13l and 13i, respectively, completely inhibit the growth of *P. aeruginosa* within T4 trophozoites. About 400 µg/mL of 13l and 13i for 24 h incubation completely inhibited the growth of *P. aeruginosa* within T7 trophozoites (Figure 3.7.).

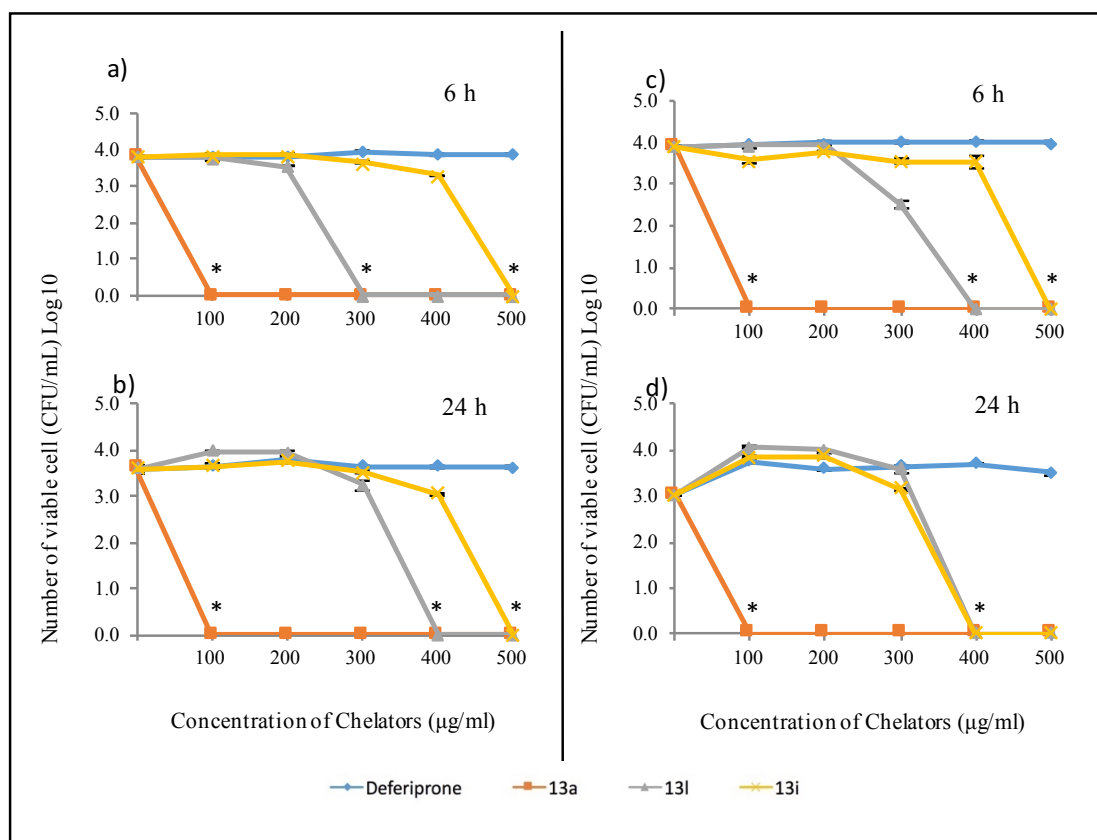


Figure 3.7 The antimicrobial effect of iron chelators on intracellular *P. aeruginosa* inside *Acanthamoeba* T4 and T7 trophozoites after treatment with different concentrations of iron chelators.

(a and b) Number of recovered *P. aeruginosa* from within *Acanthamoeba* T4 after 6 and 24 h incubation, respectively. (c and d) Number of recovered *P. aeruginosa* from within *Acanthamoeba* T7 after 6 and 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

3.3.4 Effect of iron chelators on the intracellular bacteria inside KB epithelial cell line and their cytotoxic effect

Antimicrobial assays for intracellular bacteria were performed to determine if the iron (III)- 3-hydroxypyridine-4-one chelators have antimicrobial activities against MRSA and *Pseudomonas* within KB epithelial cell line. These findings revealed that all the iron chelators tested had different ranges of antimicrobial effect on the intracellular MRSA and *P. aeruginosa* after 24 h with the lowest concentration used. Deferiprone, 13a and 13l showed similar inhibition effect at 500 µg/mL after 6 h incubations, with bactericidal rate 74.25 %, 74.25 % and 77.67 %, while 13i showed the lowest activity at the same conditions with 60.36 % bactericidal rate. After 24 h incubation, the chelators 13l and 13i exhibited a large effect on MRSA at low concentrations (100 and 300 µg/mL, respectively), with bactericidal rates of 95.82 % and 94.90 %, respectively (Figure 3.8.). While 13a and the control chelator showed low activity against intracellular MRSA after 24 h incubation at 500 µg/mL, with bactericidal rate 38.10 % and 44.57 % respectively.

The antimicrobial activities of all iron chelators against intracellular *P. aeruginosa* within KB the epithelial cell line after 6 h were low. However, the bactericidal effect of all chelators on intracellular *P. aeruginosa* were high at 200 µg/mL after 24 h incubation, with a bactericidal rate ranging between 95.04 – 97.22 % (Figure 3.8.). The MBC for all iron chelators on the intracellular bacteria for both MRSA and *P. aeruginosa* is over 500 µg/mL, as they did not completely eliminate at this concentration.

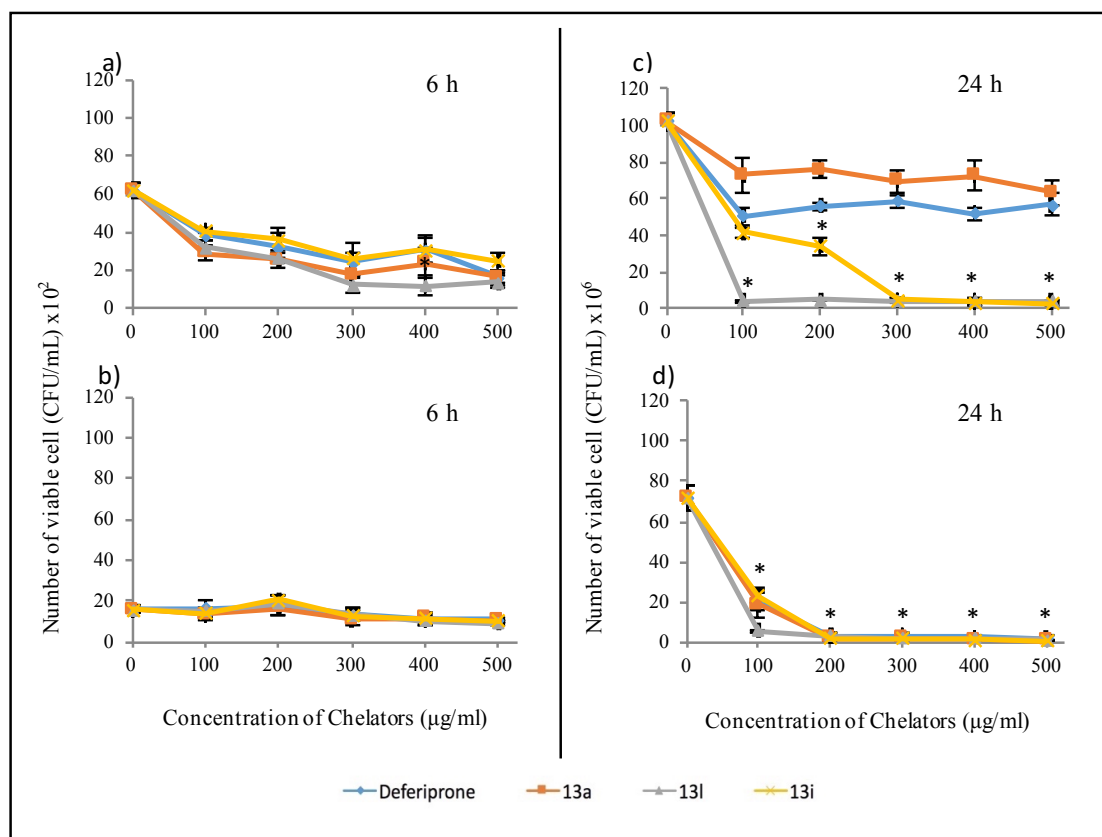


Figure 3.8 The antimicrobial effect of iron chelators on intracellular MRSA and *P. aeruginosa* inside KB epithelial cell line.

(a) Number of recovered MRSA within KB epithelial cell line after 6 h incubation. (b) Number of recovered *P. aeruginosa* within KB epithelial cell line after 6 h incubation. (c) Number of recovered MRSA within KB epithelial cell line after 24 h incubation. (d) Number of recovered *P. aeruginosa* within KB epithelial cell line after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

An LDH cytotoxicity detection kit was used to determine the toxic effect of the chelators on the KB cell line and the cytotoxic effect of bacteria on the cell line in the presence and absence of the chelators. Cytotoxicity results showed <10 % toxicity for the chelators on the cells after 6 and 24 h, which is very low (Figure 3.9.). In addition, iron chelators did not show a real reduction of bacterial toxicity after 6 h incubation, whereas the cytotoxic effect of MRSA and *P. aeruginosa* in the presence and absence of chelators were <10 % (Figure 3.10.a and Figure 3.11.a). However, MRSA showed the highest cytotoxic effect on KB cells after 24 h, of approximately 54.3 %. The lowest concentration (100 µg/mL) of 13a, 13l and 13i had reduced the cytotoxicity 13.1 %, 54.8 % and 37.7 % (respectively) to make MRSA toxicity around 47.2 %, 24.5 % and 33.8 % (respectively), while the toxicity was reduced 5.9 % by deferiprone at the same concentration. Moreover, 500 µg/mL of 13a, 13l and 13i had reduced MRSA toxicity of 49.2 %, 94.7 % and 59.1 % (respectively), compared with deferiprone which reduced the toxicity to 44.6 % (Figure 3.10.b). *P. aeruginosa*'s cytotoxicity on untreated KB epithelial cells was approximately 41.8 %. The toxic effect of *P. aeruginosa* on the cell line was decreased gradually by chelators, and at 100 µg/mL 13l had proportionately the greatest effect as it reduced the toxicity 81.8%, compared with deferiprone which just reduced the toxicity 8.8% at the same concentration. The highest concentration (500 µg/mL) of 13a, 13l and 13i showed 48.1 %, 87.9 % and 90.3 % (respectively) reduction rate of *P. aeruginosa*'s toxicity, compared with deferiprone that showed 42.3 % reduction rate (Figure 3.11.b).

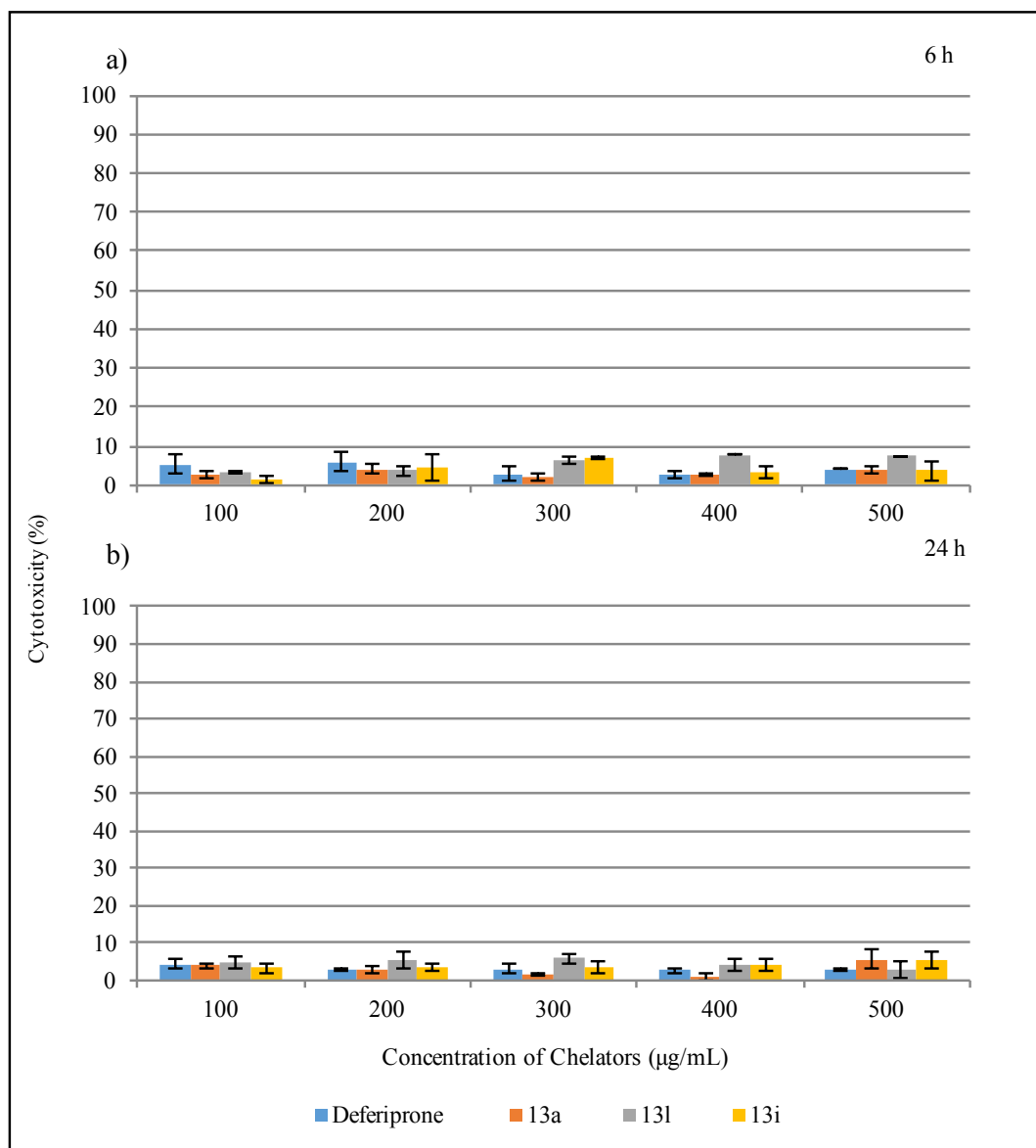


Figure 3.9 The cytotoxic effect of iron chelators on KB epithelial cell line after treatment with different concentrations of iron chelators.

(a) Percentage of iron chelators' cytotoxicity on KB epithelial cell line after 6 h incubation. (b) Percentage of iron chelators' cytotoxicity on KB epithelial cell line after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

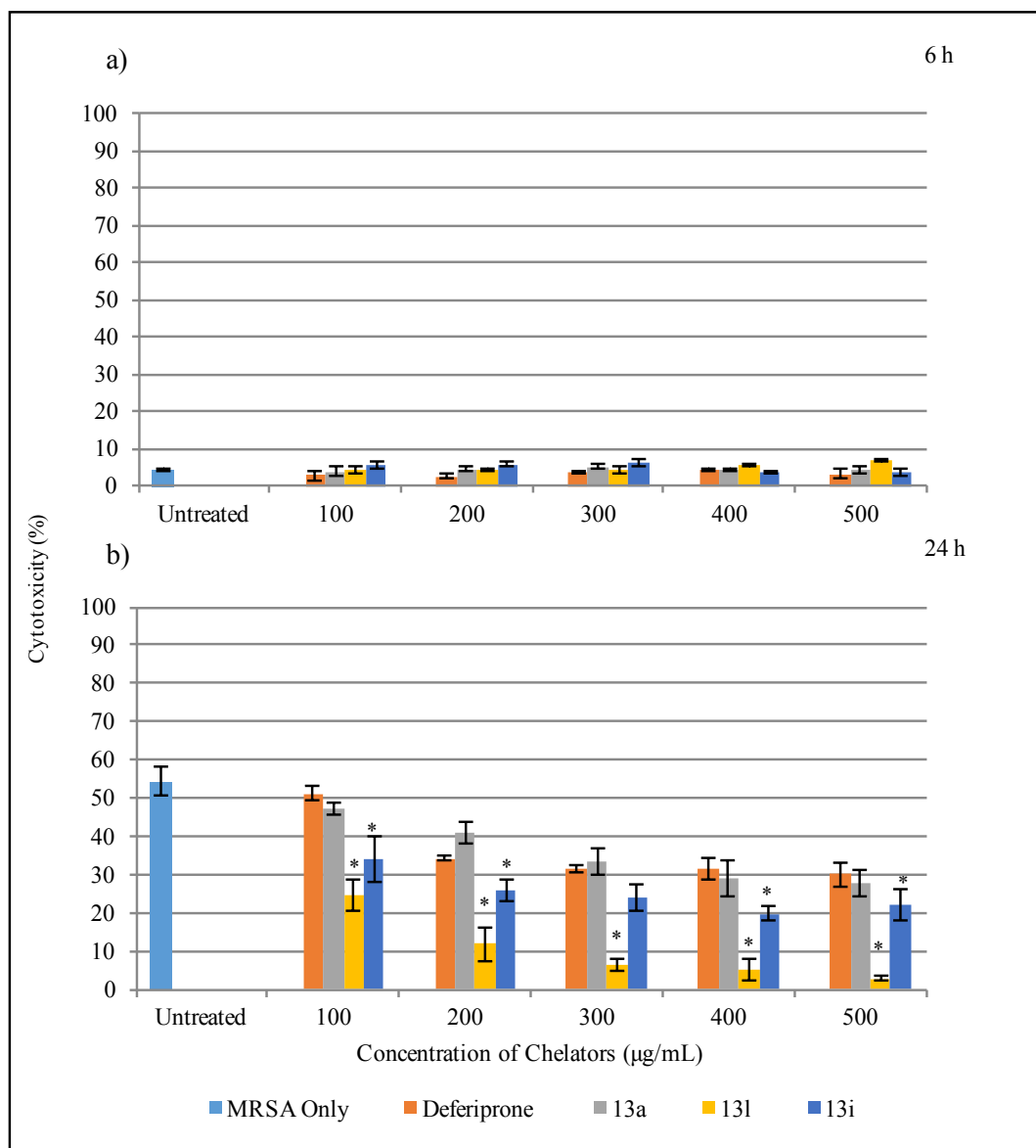


Figure 3.10 The effect of iron chelators on the cytotoxic effect of MRSA on KB epithelial cell line after treatment with different concentrations of iron chelators.

(a) Percentage of MRSA cytotoxicity on KB epithelial cell line after 6 h incubation. (b) Percentage of MRSA cytotoxicity on KB epithelial cell line after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

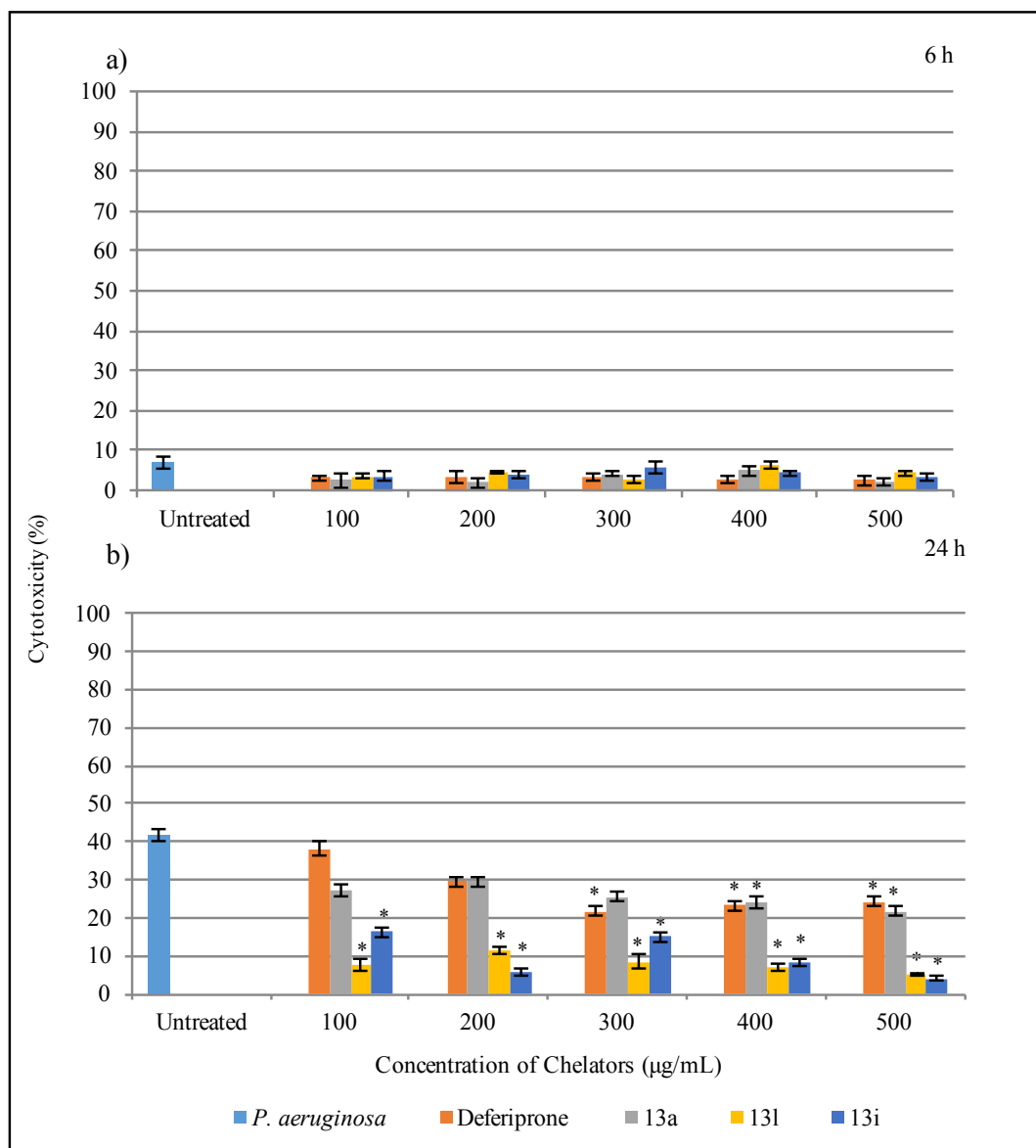


Figure 3.11 The effect of iron chelators on the cytotoxic effect of *P. aeruginosa* on KB epithelial cell line after treatment with different concentrations of iron chelators.

(a) Percentage of *P. aeruginosa* cytotoxicity on KB epithelial cell line after 6 h incubation. (b) Percentage of *P. aeruginosa* cytotoxicity on KB epithelial cell line after 24 h incubation. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

To determine the efficacy of the chelators as potent treatments, cell visualising assays were performed. Control KB cells uninfected and untreated showed intact monolayers without damages or spaces between cells, while the other controls that had been infected with MRSA or *P. aeruginosa* showed large damaged areas and disruption in the monolayer of KB epithelial cells (Figure 3.12.a and Figure 3.13.). Furthermore, the disruption caused by both MRSA and *P. aeruginosa* in the monolayer of KB epithelial cell was decreased gradually with increasing iron chelator concentrations (Figure 3.12.b and Figure 3.13.). The disruption of the monolayer of epithelial cell line infected by *P. aeruginosa* was higher than that infected by MRSA. Disruption amelioration was dose dependent; it decreased gradually with the increase in concentration of iron chelators (Figure 3.13.).

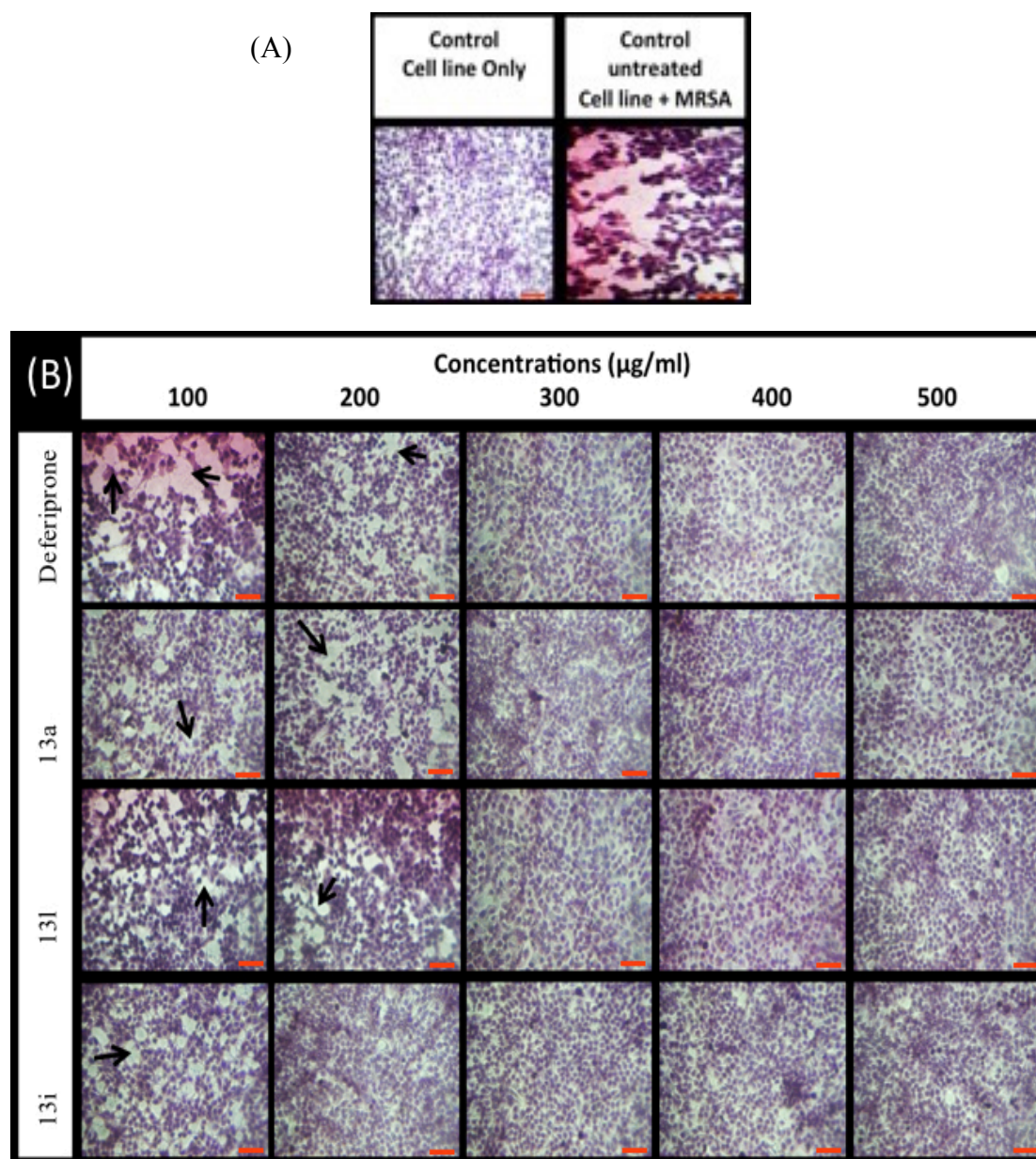


Figure 3.12 The efficacy of the chelators as potent treatments against MRSA.

(a) Positive control untreated and uninfected KB epithelial cell (Left); and negative control MRSA infected KB epithelial cells after 24 h but untreated. (b) Infected KB epithelial cell monolayer by MRSA and treated with different concentrations of deferiprone, 13a, 13l and 13i chelators. Disruption in epithelial cells by MRSA was decreased gradually with increasing concentration of the chelators. (Disruption area in the monolayer caused by MRSA are referred by arrows; bars = 50 μm).

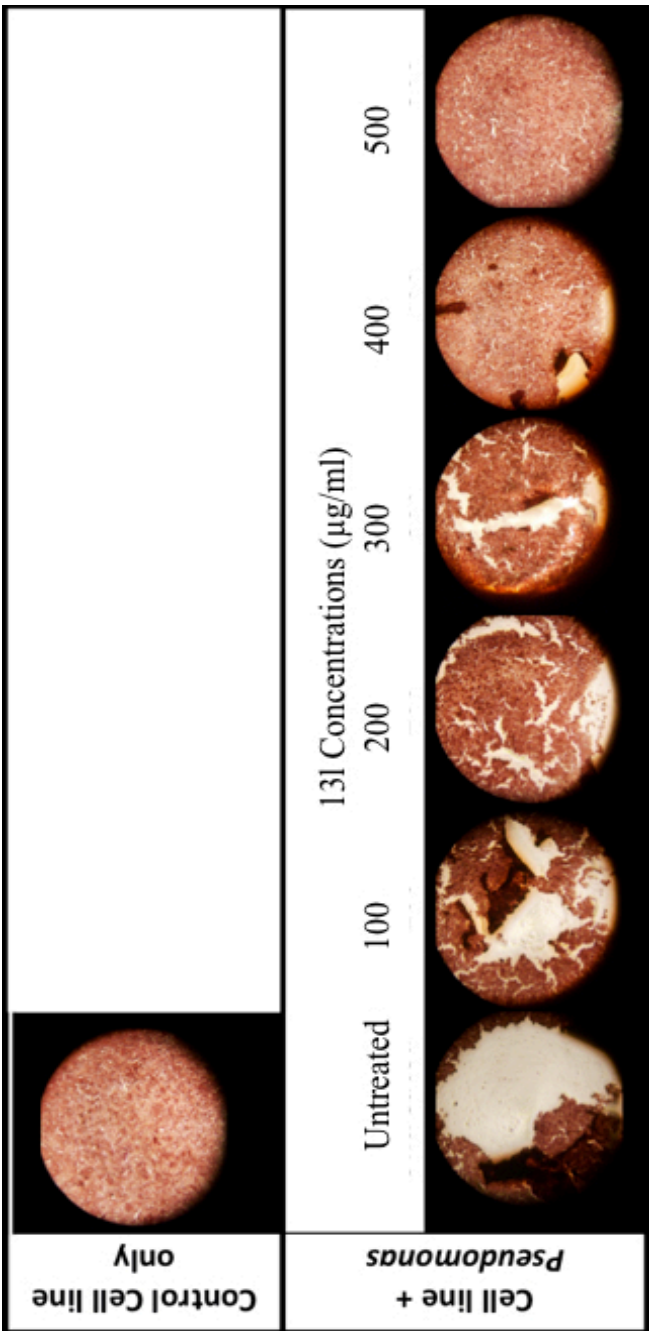


Figure 3.13 The efficacy of the chelators as potent treatments against *P. aeruginosa*.

Top image represents positive control KB epithelial cell uninfected and untreated; negative control infected KB epithelial cells by *P. aeruginosa* but untreated (Left image in the second row); and then different concentrations of 131 chelators. Disruptions on the monolayer of epithelial cell caused by *P. aeruginosa* were decreased gradually with increasing concentration of the chelators.

3.3.5 The effect of iron chelators on the expression of *Acanthamoeba* extracellular proteases

To detect the inhibitory effect of the iron (III)- selective HPO chelators on *Acanthamoeba* proteases, zymographic assays were performed. Deferiprone showed the greatest inhibition of *Acanthamoeba* proteases at 500 µg/mL compared with the other novel iron chelators. It was found that deferiprone inhibits the proteases at 115 and 100 kDa; other proteases were increased by 131 (Figure 3.14a). Four other zymographic gels were prepared to investigate the dose dependency of the iron chelators on the expression of *Acanthamoeba* proteases (Figure 3.14b, 3.14c, 3.14d and 3.14e). Increasing the concentration of 131 enhanced *Acanthamoeba* protease production (Figure 3.14c); but there was no difference between concentrations for other iron chelators.

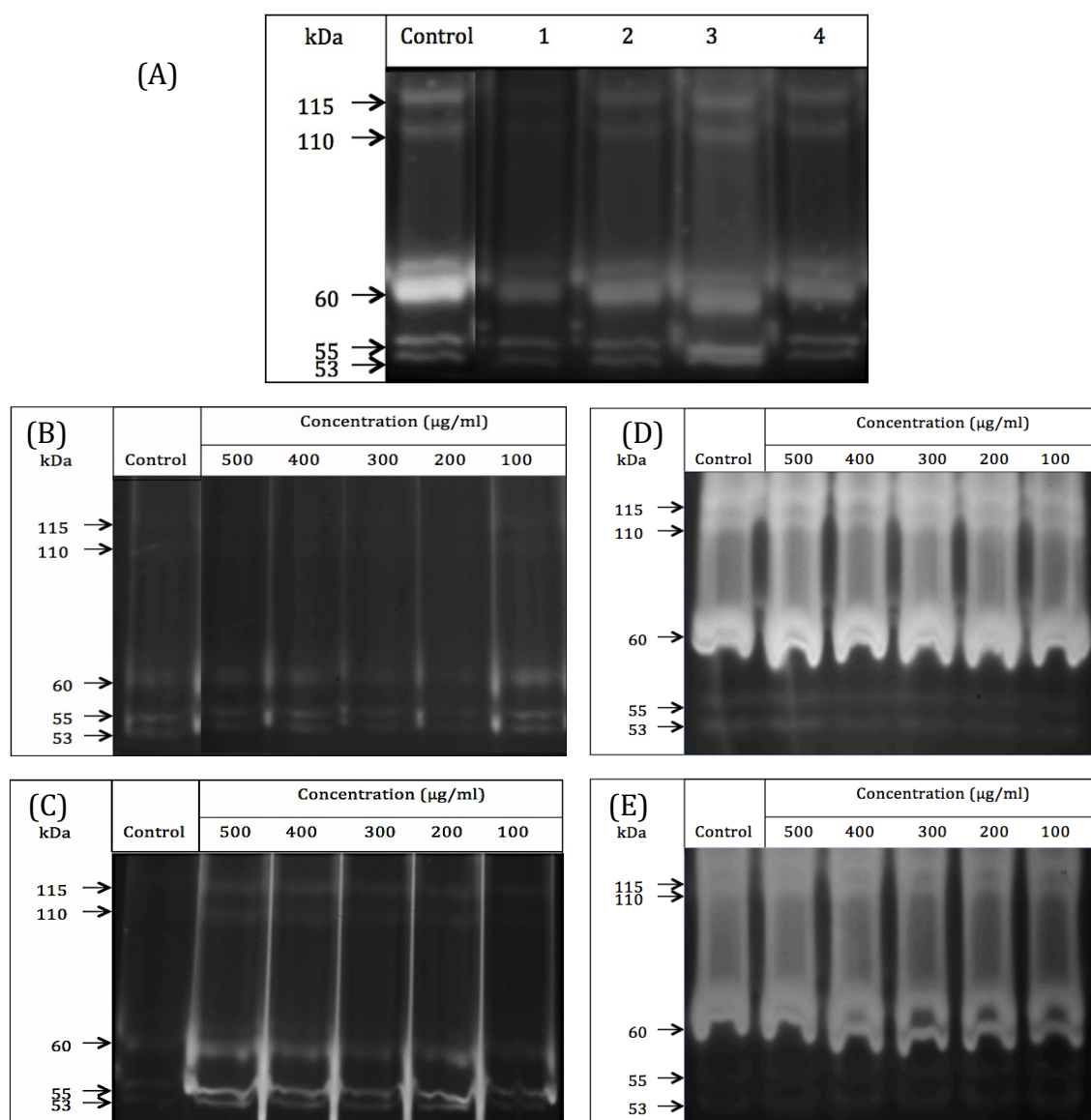


Figure 3.14 Condition media (CM) of *Acanthamoeba* T4 incubated in RPMI overnight to determine the ability of iron chelators to inhibit *Acanthamoeba* proteases.

(a) Control untreated, then CM of *Acanthamoeba* in presence of the chelators at 500 μg/mL, lane1: deferiprone, lane2: 13a, lane3: 13l, and lane4: 13i. Deferiprone were decreased proteases expression. (b) CM 5 different concentrations of deferiprone. (c) 5 different concentrations of 13l. (d) 5 different concentrations of 13a. (e) 5 different concentrations of 13i.

3.4 Discussion

Microorganisms face a continuous struggle for iron using acquisition and assimilation strategies which share several functional and mechanistic principles. One common theme is the utilization of high-affinity chelators for extracellular iron mobilization, generally known as siderophore-dependent iron acquisition. The other strategy is to release the mobilized iron from extracellular sources to allow its transfer and incorporation into metabolically active proteins. A variety of mechanisms which are often coupled with high-affinity uptake have evolved to facilitate the removal of iron from siderophore ligands; however, they differ in many key aspects including substrate specificities and release efficiencies (Hassett *et al.*, 1998; Miethke, 2013). Although heme is a valuable nutrient source for invading pathogens, the intracellular accumulation of heme is toxic due to the molecule's reactivity. Therefore, organisms that acquire exogenous heme to satisfy nutrient iron needs must have adaptable mechanisms to avoid surplus heme accumulation (Torres *et al.*, 2007). Catecholate siderophores are common in Gram-negative bacteria, such as *Pseudomonas*, and that depends on several reasons, including complex stability, high environmental pH and a weak capability for nitrogen metabolism (Winkelmann, 2002). MRSA as a Gram-positive bacterium produces two siderophores: staphyloferrin A and staphyloferrin B, and these both are synthesised via the nonribosomal peptide synthetase independent pathway (NIS) (Hammer and Skaar, 2011).

Previous studies with iron chelators have demonstrated their bactericidal effect (Qiu *et al.*, 2011; Xu *et al.*, 2011; Thompson *et al.*, 2012). Although *N,N'*-bis(2-hydroxybenzyl) ethylenediamine-*N,N'*-diacetic acid, possesses very high affinity for iron, it was found to be a weak inhibitor of bacterial growth (Chew *et al.*, 1985). In an attempt to develop drugs with comparable iron binding capacity but reduced bacterial

virulence-enhancing properties, we have investigated the effect of three novel iron chelators based on the structure of deferiprone.

Because of structurally different siderophores and different cell wall types, it can be expected that iron selective chelators will have a differential influence on a range of bacteria. The high efficacy of hydroxypyridinone iron-chelators against *Pseudomonas* strains is consistent with the outer membrane of Gram-positive bacteria. The penetration of compounds is expected to be limited by molecular mass above about 500–600 Dalton (Hancock and Nikaido, 1978). The molecular mass for deferiprone, 13a, 13i and 13l are 139.15, 739.35, 941.51 and 1025.61 Da, respectively.

Deferiprone is a very small, neutral molecule, and these properties are known to facilitate its transfer across host cell membranes and may be bacterial membranes as well. However, wound tissue is already disrupted, therefore, compounds with higher molecular mass can probably pass through cell membranes by different mechanisms, such as endocytosis (Sohn *et al.*, 2012). The compounds 13a, 13i, and 13l feature three moieties of (3-Hydroxypyridine-4-one), allowing them to form hexadentate ligands with high affinity to iron (III) by comparison with deferiprone which forms a bidentate iron complex. These novel iron chelators possess stronger inhibitory activities against the growth of both MRSA and *Pseudomonas* than deferiprone. Compound 13l conferred the highest growth inhibition, exceeding 13a and 13i. The higher efficacy of 13l could be due to its increased hydrophobicity. Compound 13l was designed with not only a diethylenglycole chain, similar to 13i compound but also has a longer hydrocarbon chain, 6(CH₂). Compound 13i has a shorter hydrocarbon chain, 4(CH₂) and compound 13a lacks either (Zhou *et al.*, 2015). Balanced hydrophobic compounds could intensify their binding characters toward protein transporting iron complexes, and also their intracellular uptake mechanisms (Górska *et al.*, 2014).

The Minimum Inhibitory Concentrations (MIC) of deferiprone on *S. aureus* and *P. aeruginosa* are (>512 $\mu\text{g/mL}$) (Thompson *et al.*, 2012); and that agrees with these results for deferiprone against MRSA and *Pseudomonas* (>500 $\mu\text{g/mL}$). There are two recent studies that used three novel iron chelators, possessing three moieties of (3-Hydroxypyridine-4-one) (Qiu *et al.*, 2011; Xu *et al.*, 2011). These compounds vary in their cores to our novel compounds. The MICs of CP251 (Qiu *et al.*, 2011) on *S. aureus* and *P. aeruginosa* are 500 and 100 $\mu\text{g/mL}$, respectively, compared to our novel chelator 13l, >500 and 400 $\mu\text{g/mL}$.

Notably, there are antiamoebic activities for all three novel iron chelators against pathogenic and non-pathogenic *Acanthamoeba*. This is the first report showing the use of iron chelation against *Acanthamoeba*. However, the use of iron chelators against amoebae had been investigated in 1983 with *Naegleria fowleri*. Antiamoebic effect of desferrioxamine B and rhodotorulic acid on *Naegleria fowleri* was found at 1000 $\mu\text{g/mL}$ after 96 h and at 2000 $\mu\text{g/mL}$ after 48 h as these concentrations completely eliminated *Naegleria* (Newsome and Wilhelm, 1983). Another study demonstrated that the survival of *Entamoeba histolytica* was affected by using iron chelators which interrupt a fermentative pathway (Espinosa *et al.*, 2009). We propose that iron chelation therapy could be used to treat *Acanthamoeba*.

Acanthamoeba T4 has the ability to protect intracellular bacteria from drugs (Abd *et al.*, 2010). However, all novel iron chelators that were used in this study have antimicrobial effect against intracellular MRSA and *P. aeruginosa* inside *Acanthamoeba* and the KB epithelial cell line. The intracellular growth of *Chlamydia psittaci*, *C. trachomatis*, and *Legionella pneumophila* inside macrophage were inhibited by using deferiprone and desferasirox (Paradkar *et al.*, 2008). 3-hydroxy-4-pyridinone hexadentate iron chelators were reported to have antimycobacterial activity against intracellular *Mycobacterium*

avium inside macrophages (Nunes *et al.*, 2010). The present findings show that MRSA and *P. aeruginosa* have toxic effects on the KB epithelial cell line. The chelators ameliorated this toxicity in a dose dependent manner. Since these chelators could have shown a toxic effect on mammalian cells, the cytotoxicity assays were performed. Fortunately, all iron chelators used in this research showed very low toxic effect on KB cells (<10%) after 24 h. However, they could be toxic on KB epithelial cell line or other types of cells if exposed for long time to these chelators. Therefore, further investigation is needed to investigate their toxicity on the same cell line and other cell lines after 24, 48 and 72 h. However, this is clearly advantageous for the use of iron chelation strategy for wound infection.

Iron could be important for *Acanthamoeba* protease production as iron has an important role in microorganism pathogenicity. However, these findings showed that there is no significant effect of iron chelators on the expression of *Acanthamoeba* proteases. In addition, deferiprone showed no effect on the viability of *Acanthamoeba*, however, it showed slightly reduction on proteases expression, which is mean that deferiprone might has an inhibitory effect on the production of proteases.

Both microorganisms themselves and their products have roles in causing infections and the induction of the immune system response. Cell death is one of these responses, which has been classified as two forms: apoptosis and necrosis. The investigation of the ability of these microorganisms to induce one or both forms of cell death, and whether cysteine-aspartic proteases (caspases) have a role in that process, is the subject of the fourth chapter.

Chapter Four

4

The role of caspase pathway in cell death of mesenchymal stem cells and skin epithelial cells induced by MRSA,
Pseudomonas and *Acanthamoeba*

Chapter 4 : The role of caspase pathway in cell death of mesenchymal stem cells and skin epithelial cells induced by MRSA, *Pseudomonas* and *Acanthamoeba*

4.1 Introduction

Chronic wounds are a serious problem around the world. The wounds which fail to heal within one month are considered as chronic wounds. Infection and vascular failure play roles in non-healing wounds. Infection caused by MRSA and *P. aeruginosa* cause complications in wounds, which lead to a prolonged inflammatory response, interfere with collagen synthesis and epithelialization, and cause damage in the tissue (Reyes-López *et al.*, 2015). Acute infection in chronic wounds can lead to biofilms forming, which could be one of the main reasons behind treatment failure (Clinton and Carter, 2015; Rondas *et al.*, 2015).

A combination of factors work together to enhance wound healing, which include growth factors such as Epidermal Growth Factor (EGF) and Vascular Endothelial Growth Factor (VEGF), Extracellular Matrix (ECM) proteins, viable epithelial cells, fibroblasts and mesenchymal stem cells (MSCs). MSCs have roles in all wound healing phases, thus their presence would support the successful healing for chronic wounds via secreting anti-inflammatory factors such as interleukin (IL-1RA) (Maxson *et al.*, 2012; Hu *et al.*, 2015). In addition, MSCs have antimicrobial activity against bacteria in wounds via two mechanisms: (i) direct by producing antimicrobial factors such as antimicrobial peptides called (LL-37) and neutrophil associated gelatinase (N-Gal), and (ii) indirect via producing immune-modulating factors which stimulate phagocytosis in the immune cells to kill bacteria (Mei *et al.*, 2010). The presence of MSCs in the skin and their important role in wound healing and the role of their antimicrobial production against bacteria suggest that using external MSCs could be a promising solution to treat

infected non-healing wounds (Paquet-Fifield *et al.*, 2009; Krasnodembskaya *et al.*, 2010; Sellheyer and Krahl, 2010).

Although cell death is a fundamental regulator of development in eukaryotic cells, it is also considered as a critical immune defence mechanism in multicellular organisms by removing infected cells to save other uninfected cells. Two main cell death modes are known: apoptosis and necrosis (Bergsbaken *et al.*, 2009; Lamkanfi and Dixit, 2010; Zitvogel *et al.*, 2010). Apoptosis induced by bacterial infections is considered as an innate immune response, as this type of cell death has two defensive roles in bacterial infections. The first role is to get rid of bacteria in the early stage of infections; while the second role is to induce immune cells to pick up apoptotic bodies which contain bacteria, then induce a protective immune response (Elliott and Ravichandran, 2010). By contrast necrosis induced by bacterial infections would allow the bacteria to go out of necrotic cells and infect neighbouring cells. The bacterial pathogens which are able to invade and multiply within host cells, use different mechanisms to control survival and death pathways of host cells to support their own ability to survive and replicate (Behar *et al.*, 2010; Kim *et al.*, 2010; Lamkanfi and Dixit, 2010). The apoptosis process is mainly mediated by intracellular cysteine-aspartic proteases (caspases), which interfere with different cellular proteins leading to demise of the cell (Haslinger-Löffler *et al.*, 2005).

4.1.1 Cysteine-aspartic proteases (Caspases)

Caspases are a family of proteases which regulate cell death and inflammation. Caspases are endoproteases which break down peptide bonds on active sites at cysteine residues (Riedl and Shi, 2004). Caspases have been classified into two groups depending on their roles. The first group contains caspases which have roles in apoptosis, including caspase-3, 6, 7, 8, and 9 in mammals. The other group contains

those which have roles in inflammation, including caspase-1, 4, 5, 12 in humans and caspase-1, 11, and 12 in mice. Moreover, those caspases, which have roles in apoptosis, have been sub-classified with regard to their mechanisms of action: either as executioner caspases (caspase-3, 6, and 7) or initiator caspases (caspase-8 and 9) (McIlwain *et al.*, 2013).

4.1.2 The extrinsic pathway of apoptosis

The extrinsic pathway is so called as it is the responsible pathway causing apoptosis by external causative agents such as, microorganisms. This pathway is stimulated by extracellular signals in the form of ligands binding to Death Receptors (DRs). These receptors are members of the Tumour Necrosis Factor (TNF) family, including TNF receptor-1 (TNFR1), Death Receptor 3 (DR3), TNF-Related Apoptosis-Inducing Ligand Receptor-1 (TRAIL-R1), and TRAIL-R2. Death receptor ligands such as TNF, TRAIL (also known as Apo2-L), and TNF-like ligand 1A (TL1A), bind to DR activating the monomeric procaspase-8 protein via death domain, and then activate caspase-8 monomers, which activate caspase-3 which induce programmed cell death (McIlwain *et al.*, 2013).

4.1.3 MRSA-induced cell death

MRSA induce apoptosis and necrosis by producing haemolysin α -toxin (Essmann *et al.*, 2003; Haslinger-Löffler *et al.*, 2005). The ability of *S. aureus* to induce apoptosis was investigated in different types of cells such as epithelial cells, endothelial cells, keratinocytes, lymphocytes and macrophages (Jonas *et al.*, 1994; Kahl *et al.*, 2000; Menzies and Kourteva, 2000; Nuzzo *et al.*, 2000; da Silva *et al.*, 2004). In order for *S. aureus* to invade host cells, they produce toxins (e.g. α -toxin) forming pores in the host cell membrane prior to invasion. However, apoptosis might not be triggered by a single staphylococcal virulence factor, but requires combinations of factors together such as

Fibronectin-Binding Proteins (FnBPs) (Peacock *et al.*, 2002). Toxic Shock Syndrome Toxin-1 (TSST-1) produced by *S. aureus* and MRSA has been demonstrated to induce apoptosis in T lymphocytes via stimulating the release of IL-1, IL-2 and TNF (Mateo *et al.*, 2007).

4.1.4 *Pseudomonas aeruginosa*-induced cell death

The ability of *P. aeruginosa* to induce apoptosis in host cells has been shown by several studies. Direct interaction of *P. aeruginosa* with host cells and secreted factors (e.g. pyocyanin) seem able to induce cell death in macrophages, neutrophils and epithelial cells (Broquet and Asehnoune, 2015). The major known virulence factor of *P. aeruginosa*-induced apoptosis in eukaryotic host cells is type III secretion system (T3SS). The T3SS is activated during contact with host cells membrane resulting in cell death or alterations in the host immune responses. This secretion system is responsible for producing four effector toxins of *P. aeruginosa* including ExoS, ExoT, ExoU and ExoY. Each exotoxin has a specific function: ExoS causes cell death and ExoU causes cell lysis. It is rare to find one strain producing all these four toxins (Galle *et al.*, 2012a, 2012b). Quorum sensing molecules which regulate virulence factors such as toxins, exotoxin A and pyocyanin, play a role in apoptosis (Rutherford and Bassler, 2012). In conclusion, T3SS along with other virulence factors produced by *P. aeruginosa* are involved in apoptosis by different mechanisms such as the generation of Reactive Oxygen Species (ROS) and the activation of the caspase pathways (Broquet and Asehnoune, 2015).

4.1.5 *Acanthamoeba*-induced cell death

A few studies reviewed the ability of *Acanthamoeba* to induce apoptosis in the host cells. Both *Acanthamoeba* and host determinants (e.g. IL-8 and TNF- α) play a role in inducing apoptosis in host cell (Siddiqui and Khan, 2012). The ability of *Acanthamoeba*

trophozoites or their lysate to induce apoptosis in human corneal epithelial cells have been demonstrated (Zheng *et al.*, 2004). In another study, the co-cultivation of *Acanthamoeba* with mouse neuroblastoma cells showed signs of apoptosis including cell shrinkage, nuclear condensation and DNA fragmentation. Caspase enzymes and mitochondrial pro-apoptotic proteins (Bax and Bcl-2) were determined in *Acanthamoeba*-mediated apoptosis (Chusattayanond *et al.*, 2010). Ecto-ATPases are one of the direct virulence factors produced by *Acanthamoeba*. These enzymes hydrolyse the extracellular ATP to ADP which have toxic effects on the host cells. Once *Acanthamoeba* binds the host cells, ADP will be released causing caspase-3 activation and then apoptosis (Khan, 2009).

Herein the role of caspase pathway in KB epithelial cells and MSC cell death induced by live, heat-killed and conditioned medium of MRSA and *P. aeruginosa*, and also *Acanthamoeba* conditioned medium is described.

4.2 Materials and Methods

4.2.1 Cell Lines and cell culture

Human KB epithelial cell line and murine Mesenchymal Stem Cells (mMSCs) were used in this study. mMSCs primary cells was provided by Dr Ralf Zwacka (School of Biological Science, University of Essex). Dulbecco's Modified Eagle Medium (DMEM)-low glucose supplemented with 20% FBS (Invitrogen) was used for mMSCs culturing and seeding. Both cell types were cultured and seeded as described in section (2.2.1).

4.2.2 Bacterial culture and conditioned media

4.2.2.1 *Live bacteria*

MRSA and *P. aeruginosa* were cultured onto CLED plates overnight. A few colonies were inoculated in LB broth and the ODs were measured using spectrophotometer as described in section (2.2.3).

4.2.2.2 *Heat-killed bacteria*

Heat-killed bacteria were prepared to investigate whether the bacterial body has role on cell death. Both strains were refreshed onto CLED plates and incubated overnight. Then, a few colonies were inoculated in LB broth and incubated for overnight at 37 °C in air incubator. OD was measured using spectrophotometer as described in section (2.2.3). Then, 1 mL of bacterial broth (containing 10⁸ CFU) was heated in a water bath at 65 °C for 90 min. About 20 µL of heat-killed bacteria were cultured onto CLED plates and incubated overnight at 37 °C in air to determine whether bacterial strains were still alive, and no bacterial growth was detected after incubation (Worgall *et al.*, 2002).

4.2.2.3 Conditioned medium from bacteria

Bacterial conditioned media (BCM) were prepared to investigate the ability of enzymes, toxins and proteases which were produced by MRSA and *P. aeruginosa* to have any role in apoptosis. Bacterial strains were inoculated in the cell media (EMEM for KB epithelial cell line; and DMEM for mMSCs) in tubes. Then, the tubes were incubated at 37 °C in air overnight. After incubation, tubes were centrifuged at 5000 x g for 10 min and then supernatants were filtered by using syringe and filter pore size (0.20 µm) to get conditioned media which contained the proteins and external virulence factors from both strains. BCMs were stored at -20 °C till required (Iqbal *et al.*, 2014).

4.2.3 Conditioned medium (CM) of *Acanthamoeba*

Acanthamoeba Conditioned Medium (CM) was prepared as described in section 3.2.6.2.1 (Sissons *et al.*, 2006; Iqbal *et al.*, 2014).

4.2.4 General caspase inhibitor

In order to investigate the role of caspase pathway in cell death induced by infection, an irreversible caspase-3 inhibitor (Z-VAD-FMK) (1 mg, purchased from BD Pharmingen) was used to inhibit caspase pathways. Stock solution (10 mM) of Z-VAD-FMK was prepared by adding 214 µL of dimethyl sulfoxide (DMSO) into the original tube containing 1 mg of the inhibitor (Worgall *et al.*, 2002).

4.2.5 Induction of cell death

To study the ability of live, heat-killed and BCM of MRSA and *P. aeruginosa*, and CM of *Acanthamoeba* to induce cell death, fluorescence (flow cytometry assays) and colorimetric (LDH assays) techniques were performed. In brief, cells were seeded and

grown in two 24 well-plates until confluent as described in sections 2.2.1.2 and 2.2.4. Then, the growth medium was discarded, and cells were washed once with PBS. To analyze whether bacteria and *Acanthamoeba* induced cytotoxicity can be influenced by caspase inhibition, one of the plates was used with caspase inhibitor (15 μ M, final concentration for 60 min pre-induction), but the other was not. Next, live, heat-killed and BCM for bacteria and *Acanthamoeba* CM were added to different wells to induce cell death. Plates were incubated for 60 min at 37 °C in humidified 95 % air, and 5 % CO₂ incubator. Next, wells with live bacteria were treated with antibiotics as described in section (2.2.6) to kill extracellular bacteria. Then plates were incubated for 24 h at 37 °C in humidified 95 % air, and 5 % CO₂ incubator. After cell death induction, cells were harvested and collected with the supernatants in 1.5 mL centrifuge tubes for flow cytometry assays. While supernatants only were collected in 1.5 mL centrifuge tubes for LDH measurement (Worgall *et al.*, 2002).

4.2.6 Flow cytometry assays (apoptosis)

Samples containing supernatants and cells were centrifuged at 1500 x g for 5 min. Then, cells were stained according to recommended method by manufacturer. Annexin V method were used to detect apoptosis and necrosis. The fixable Zombi NIR were used instead of Propidium Iodide (PI) and 7-AAD in these assays. Briefly, pellets of cells were washed twice with 200 μ L of cold cell staining buffer (BioLegend). Then, cells (1.0×10^5) were resuspended in 100 μ L of Annexin V binding buffer (BioLegend) containing 5 μ L of Annexin V FITC (BioLegend) and 1 μ L of Zombie NIR (BioLegend). Samples were gently vortexed and incubated at room temperature (RT) for 20 min in the dark. Next, samples were washed once with cell staining buffer and fixed with 4% paraformaldehyde (PFA) on ice for 20 min. Then, all samples were

centrifuged and supernatants were discarded. Pellets were washed once with cell staining buffer and then resuspended in 400 μ L of Annexin V binding buffer. Next, cells were analysed by flow cytometry [The BD Accuri™ C6] and each run was restricted to 10000 moments.

4.2.7 Lactate dehydrogenase (LDH) assays (necrosis)

LDH assays were performed to detect the role of caspase pathway in cell damage caused by live, heat-killed and BCM of MRSA and *P. aeruginosa*, and CM of *Acanthamoeba* on both KB cell line and mMSC cells. Samples prepared in section (4.2.5) were used for these assays. LDH assays were performed as described earlier in section (3.2.8.2) (Worgall *et al.*, 2002; Chan *et al.*, 2013).

4.2.8 Software and statistics

FlowJo software (v10.1) was used to analyse and prepare flow cytometry graphs. In addition, Microsoft office Excel programme was used to prepare all bar charts and analyse data, as described in section (2.2.7).

4.3 Results

4.3.1 Induction of cell death by bacteria at different time intervals

To study the effects of MRSA and *P. aeruginosa* in wound healing, cell death induction by MRSA and *P. aeruginosa* was determined. Cell death induced by MRSA and *P. aeruginosa* was determined at different time intervals to investigate the induction of apoptosis and then necrosis. KB and mMSC were infected with ~10 CFU/cell for 4, 6 and 12 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Cells with double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells staining positive for annexin V-FITC and negative for Zombie NIR were apoptotic cells. Live cells are those cells with low Zombie NIR and negative for annexin V-FITC. Percentages of apoptotic and necrotic cells of KB epithelial cell line were slightly and gradually increased after being infected with MRSA, while they significantly increased in mMSC to be 23 ± 10 % for necrotic cells and 8 ± 3 % for apoptotic cells at 12 h post-infection. Cell death induced by *P. aeruginosa* was somewhat higher than cell death induced by MRSA in both KB epithelial and mMSC cells. The percentage of necrotic KB epithelial cells induced by *P. aeruginosa* was 35 ± 2 % at 12 h post-infection, while for the apoptotic cells it was 13 ± 1.5 % at the same time. In mMSC, *P. aeruginosa* were able to induce both cell death forms, the percentage of necrotic cells after 12 h infection was about 28 ± 2 % and apoptotic cells about 8 ± 2 % (Figure 4.1 and Figure 4.2).

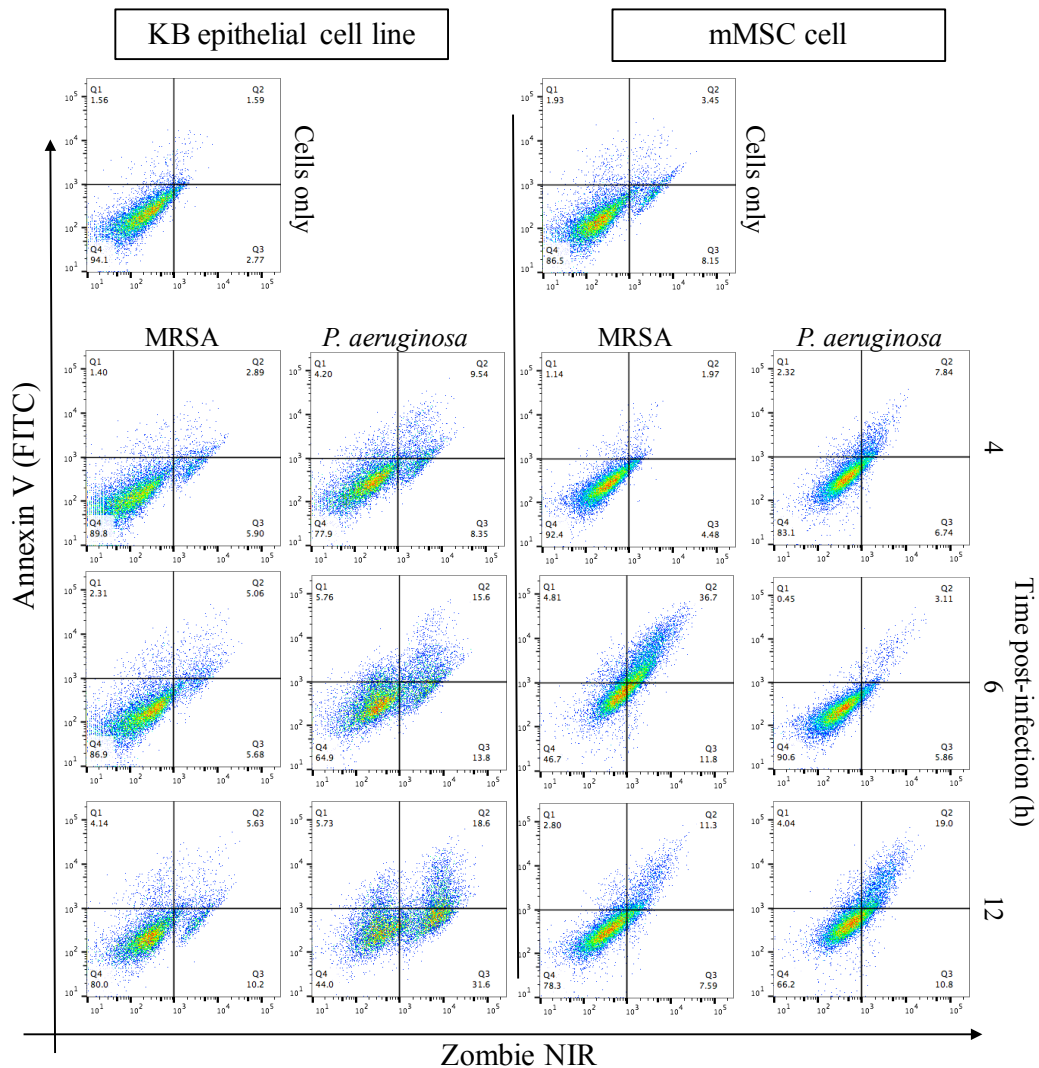


Figure 4.1 Evaluation of apoptosis in KB epithelial and mMSC cells after exposure to MRSA and *P. aeruginosa*.

Both cell types KB and mMSC were infected with 10 CFU/cell for 4, 6 and 12 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Cells with double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells with positive annexin V-FITC and negative Zombie NIR were apoptotic cells. Live cells were those cells with low Zombie NIR and negative for annexin V-FITC. Controls (cells only) showed high percentage of viable cells with very low apoptotic and necrotic cells. Apoptotic and necrotic cells increased gradually in both host cells types after increasing exposure time to bacterial strains. The data shown are representative of four experiments.

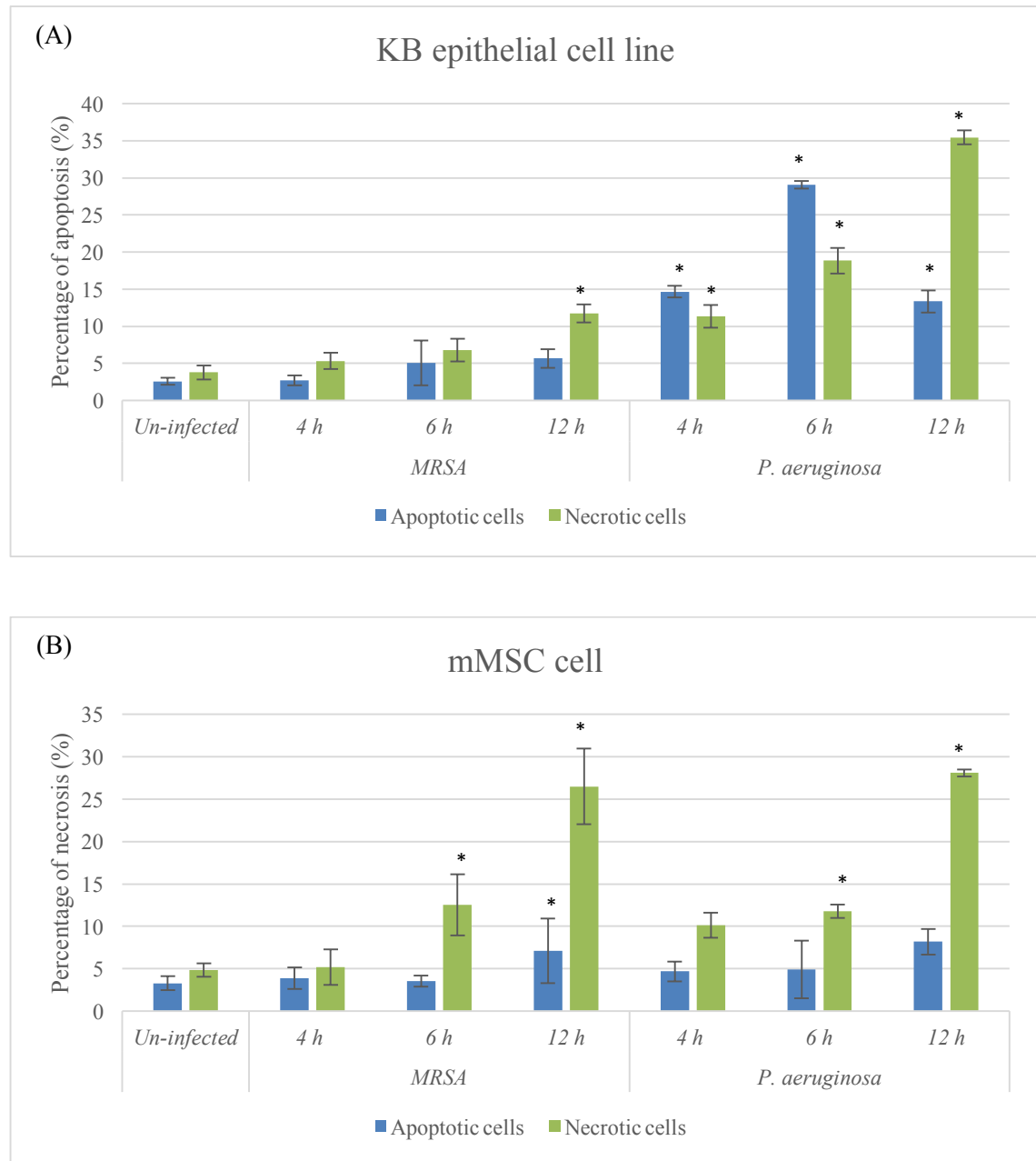


Figure 4.2 Evaluation of apoptosis and necrosis in KB epithelial cell line and mMSC cells after exposure to MRSA and *P. aeruginosa*.

Both cell types were infected with 10 CFU/cell for 4, 6 and 12 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Controls (cells only) showed low percentages of low apoptotic and dead cells. Apoptotic and necrotic cells increased gradually in mMSC cells after increasing exposure time to both bacterial strains, while apoptotic cells were increased then decrease in KB epithelial cells at 12 h post-infection. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

4.3.2 Role of caspase pathway in MRSA-induced cell death

The Role of the caspase pathway in MRSA-induced cell death was determined by fluorescence and colorimetric techniques. Cells with and without Z-VAD-FMK inhibitor were infected by one of three different conditions of MRSA: including live, heat-killed bacteria, and BCM for 24 h. Then, cell death was determined by flow cytometry after staining with annexin V-FITC and Zombie NIR. To quantify the necrotic cells, LDH released from necrotic cells was measured by colorimetric assay.

The percentage of viable KB epithelial cells after being infected with live MRSA was 10 ± 2 %, while with caspase inhibitor the viable cells increased to 34 ± 5 % (Figure 4.3 and Figure 4.4a). The reduction of necrosis-induced by live MRSA after using caspase inhibitor was 16 %, whereas they dropped from 79 ± 5 % to 66 ± 5 % (Figure 4.4b). The total apoptotic cells induced by heat-killed MRSA was about 86.1 % without Z-VAD-FMK inhibitor, while the percentage dropped to 55.5 % after inhibiting caspase pathway (Figure 4.3). Necrosis was inhibited as well under the same conditions, with their percentage about 55 % lower after using inhibitor (Figure 4.4b), while the viable cells jumped over 40 % from 14 ± 1.5 % to be 44 ± 1.5 % (Figure 4.4a). Cell death induced by BCM was about 90 % without inhibiting caspase pathway, while this number decreased to be about 60 % with caspase inhibitor (Figure 4.3). The viable cells of the KB epithelial line were increased by inhibiting caspase pathway from 12 ± 5 % to be 37 ± 3 % (Figure 4.3 and Figure 4.4a), while the necrotic cells decreased approximately 12 % by using the inhibitor to be 55 ± 6 % (Figure 4.4b).

The percentage of viable mMSC cells were increased with using Z-VAD-FMK inhibitor from 8 ± 1.5 % to 31 ± 5 % after infected by live MRSA (Figure 4.3 and Figure 4.4a), while necrotic cells were slightly decreased from 68 ± 3 % to 57 ± 10 % (Figure 4.4b). mMSC cells infected by heat-killed MRSA showed reduction in the apoptotic cells

when the caspase pathway inhibited to be 31 %, while they were about 49 % without inhibitor (Figure 4.3). The necrotic cells after inhibiting caspase pathway were decreased from 15 ± 1 % to 8 ± 2 %; nevertheless, the viable cells were increased 15 % to be 78 ± 2 %. The MRSA BCM showed cell death induction on the mMSC cells after 24 h post-infection. Caspase inhibitor reduced cell death from 70 % to 60 %. The viable cells were increased when caspase pathway inhibited to be 39 ± 4 %, compared with 24 ± 3 % without inhibitor (Figure 4.3 and Figure 4.4a).

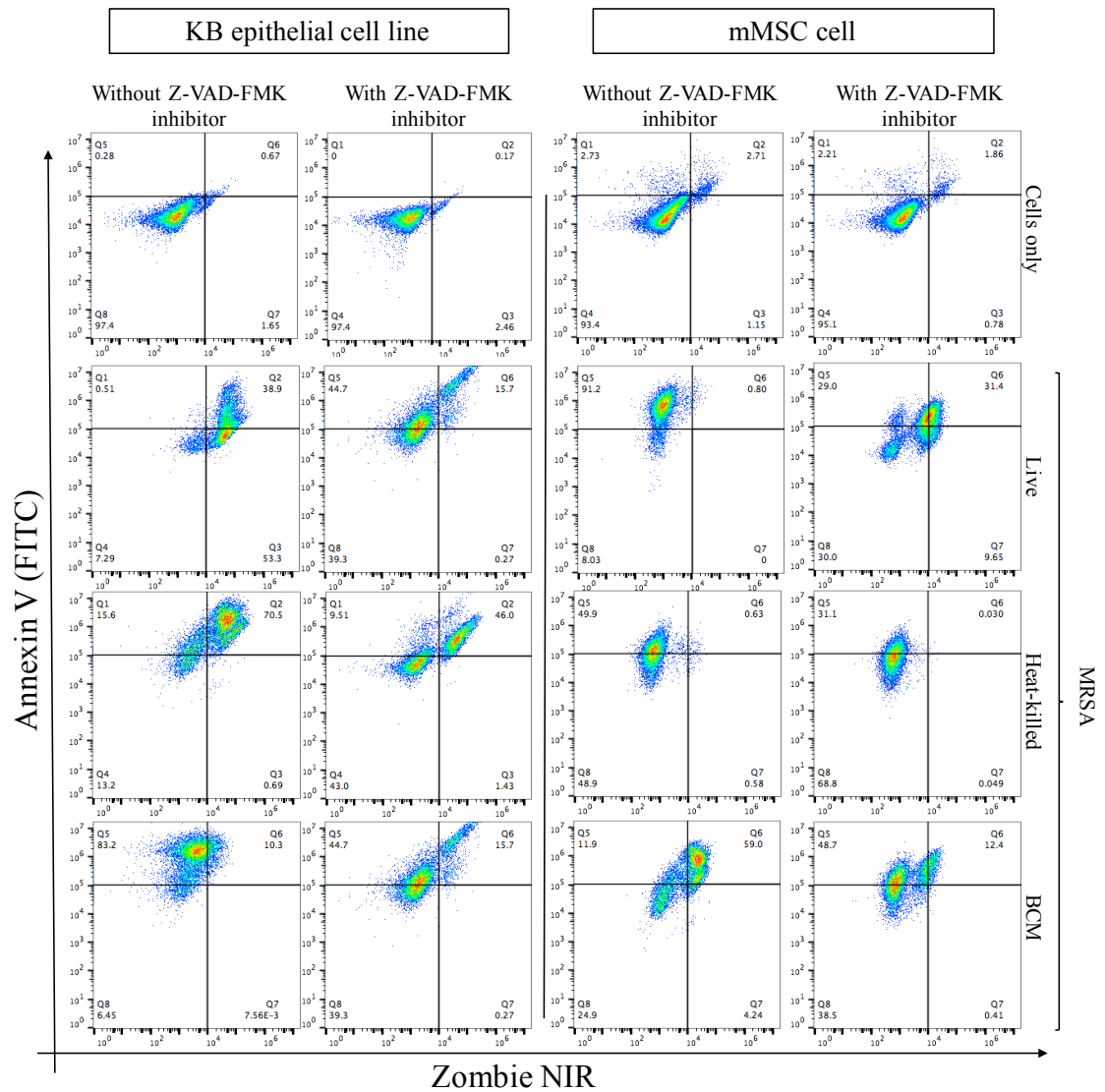


Figure 4.3 Evaluation of caspase pathway role in apoptosis induced by MRSA on KB epithelial cell line and mMSC cells.

Both cell types were infected with live or heat-killed bacteria (10 CFU/cell) or BCM of MRSA for 24 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Cells with double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells with positive annexin V-FITC and negative Zombie NIR were apoptotic cells. Live cells were those cells with low Zombie NIR and negative annexin V-FITC. Controls (cells only) showed high percentage of viable cells with very low apoptotic and dead cells. Cells infected by live bacteria and BCM showed very low number of viable cells with increasing on the number of apoptotic and dead cells compared with those cells infected with heat-killed. Live cells were increased after using Z-VAD-FMK inhibitor in both host cells types. Shown is a representative of four experiments.

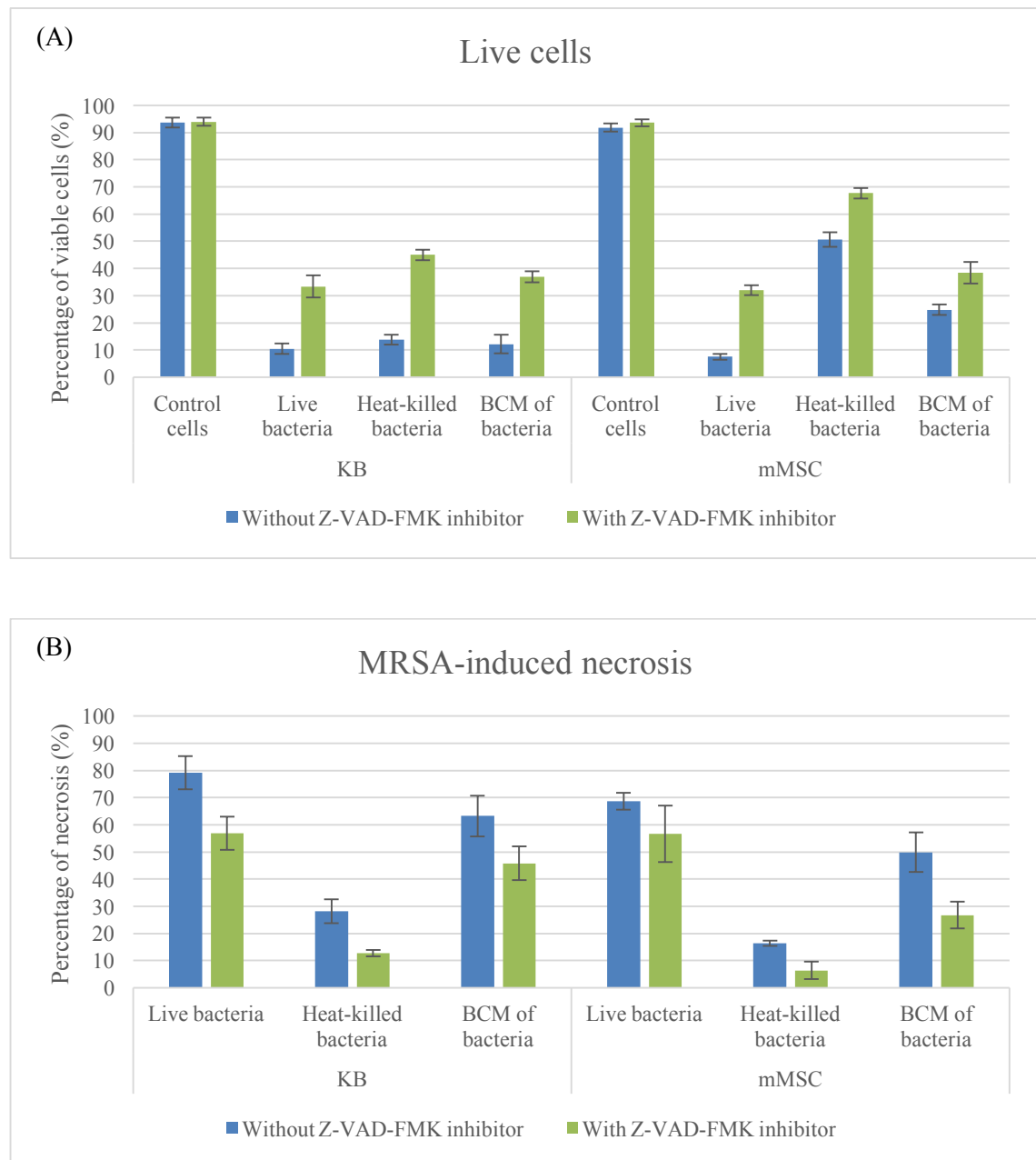


Figure 4.4 Evaluation of caspase pathway role in cell death induced by MRSA on KB epithelial cell line and mMSC cells.

Both cell types were infected with live or heat-killed bacteria (10 CFU/cell) or BCM of MRSA for 24 h. (A) Quantification of viable cells following exposure to live or heat-killed bacteria (10 CFU/cell) or BCM of MRSA for 24 h with and without Z-VAD-FMK inhibitor assessed by annexin V-FITC and Zombie NIR by flow cytometer (- annexin V-FITC/ - Zombie NIR). (B) Quantification of necrotic cells following exposure to live or heat-killed bacteria (10 CFU/cell) or BCM of MRSA for 24 h with and without Z-VAD-FMK inhibitor assessed by measuring LDH release into the supernatant using a colorimetric assay. Viable cells were increased as a result of apoptotic cells reduction by inhibit caspase pathway. Necrotic cells were slightly decreased when caspase pathway inhibited. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

4.3.3 Role of caspase pathway in *P. aeruginosa*-induced cell death

Role of caspase pathway in *P. aeruginosa*-induced cell death was determined by fluorescence and colorimetric techniques. Caspase inhibitor (Z-VAD-FMK) had been used to investigate caspase pathway role in *P. aeruginosa* infections. Three different conditions of *P. aeruginosa*: including live, heat-killed bacteria, and BCM for 24 h were used in this study. Viable and apoptotic cells were determined by flow cytometer after stained with annexin V-FITC and Zombie NIR, while necrotic cells were measured by determine the amount of LDH released from necrotic cells by colorimetric assays.

The percentage of viable KB epithelial cells post-infected with live *P. aeruginosa* was 5 ± 3 %, while the percentage of viable cells with using caspase inhibitor was increased to be 30 ± 3 %. At the same condition, the percentage of apoptotic cells was decreased when caspase inhibitor was used from 94 % to be 39 %, while necrotic cells was decreased from 80 ± 5 % to be 37 ± 8 % after inhibiting caspase cascade (Figure 4.5 and Figure 4.6a). Necrotic cells were dropped from 80 ± 5 % to 37 ± 8 % after using caspase inhibitor (Figure 4.6b). Z-VAD-FMK inhibitor reduced apoptotic KB epithelial cells induced by heat-killed *P. aeruginosa* from 44 % to 25 % (Figure 4.5), while the necrotic cells was slightly decreased from 18 ± 1 % to 8 ± 3 % (Figure 4.6b). Therefore, the viable cells were jumped from 34 ± 4 % to 73 ± 6 % (Figure 4.5 and Figure 4.6a). Apoptosis induced by BCM of *P. aeruginosa* was about 73 % without inhibiting caspase pathway, while this number decreased to be about 38 % with caspase inhibitor (Figure 4.5). The percentage of necrotic KB epithelial cells was decreased by inhibiting caspase pathway from 44 ± 7 % to be 25 ± 3 % (Figure 4.5 and Figure 4.6a), while the viable cells were increased from 33 ± 4 % to 52 ± 6 % (Figure 4.6b).

Percentage of viable mMSC cells was slightly increased with using Z-VAD-FMK inhibitor from 13 ± 3 % to 22 ± 5 % after infected by live *P. aeruginosa* (Figure 4.5 and Figure 4.6a), while necrotic cells was decreased from 70 ± 7 % to 43 ± 7 % (Figure 4.6b). While heat-killed *P. aeruginosa* showed significantly reduction on the apoptotic cells when the caspase cascade blocked to be 25 %, which it was 70 % without inhibitor (Figure 4.5). The necrotic mMSC cells after inhibiting caspase pathway and infected with heat-killed *P. aeruginosa* was decreased 50 % to be 21 ± 2 %, while the viable cells was increased from 30 ± 5 % to 78 ± 2 % (Figure 4.6a,b). The *P. aeruginosa* BCM showed increasing in the viable mMSC cells when the caspase cascade inhibited from 72 ± 2 % to 80 ± 3 %. Caspase inhibitor reduced cell death induced by BCM, whereas the dead cells was 20 % to be 11 %. The percentage of necrotic cells was decreased when caspase pathway inhibited to be 12 ± 2 %, compared with 30 ± 7 % without inhibitor (Figure 4.5 and Figure 4.6a).

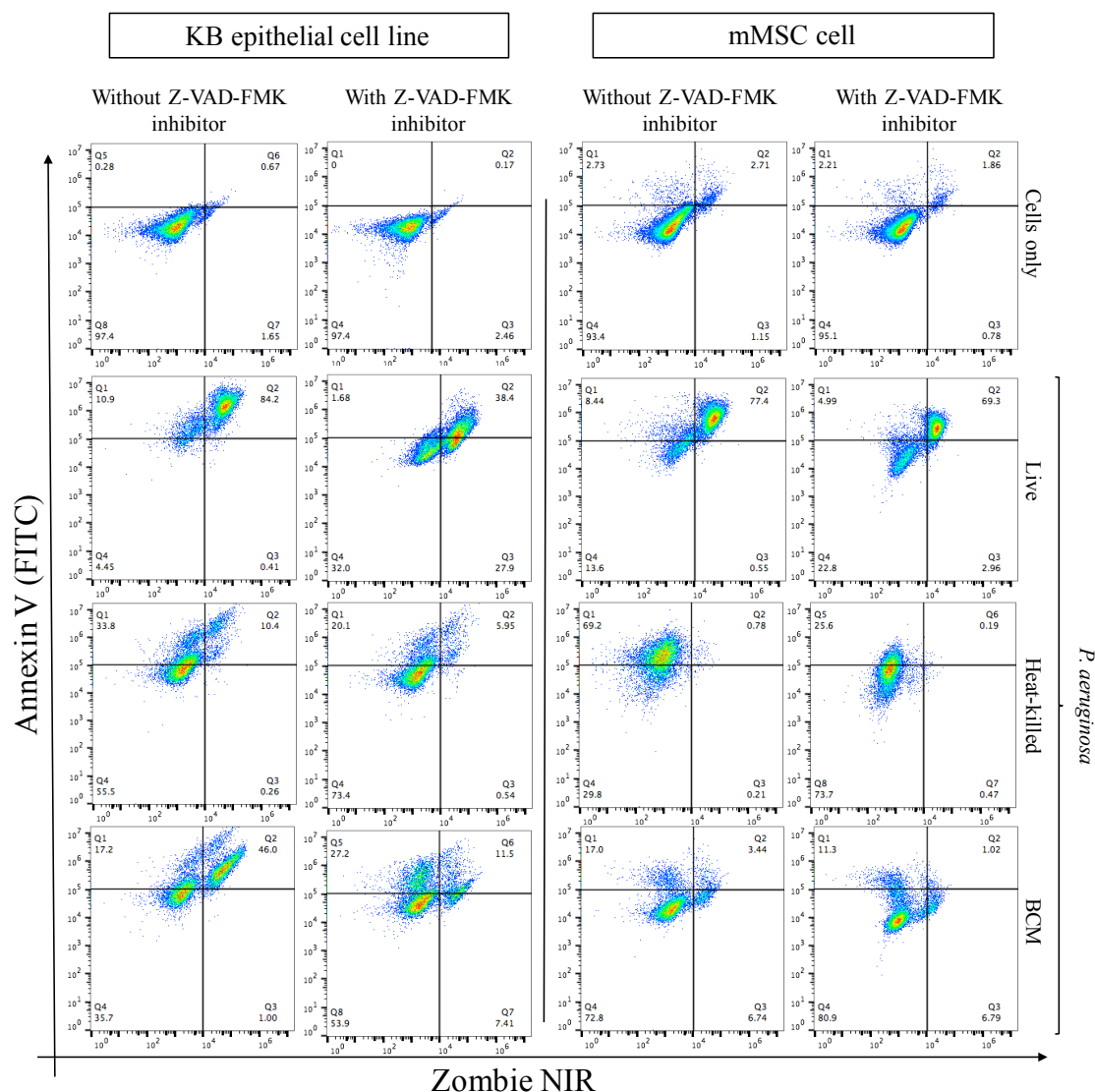


Figure 4.5 Evaluation of caspase pathway role in apoptosis induced by *P. aeruginosa* on KB epithelial cell line and mMSC cells.

Both cell types were infected with live or heat-killed bacteria (10 CFU/cell) or BCM of *P. aeruginosa* for 24 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Cells with double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells with positive annexin V-FITC and negative Zombie NIR were apoptotic cells. Live cells were those cells with low Zombie NIR and negative annexin V-FITC. Controls (cells only) showed high percentage of viable cells with very low apoptotic and dead cells. Cells infected with live bacteria showed very low number of viable cells with increasing on the number of apoptotic and dead cells compared with infected cells by heat-killed bacteria and BCM of *P. aeruginosa*. Apoptotic and dead cells were decreased after using Z-VAD-FMK inhibitor in both host cells types. Shown is a representative of four experiments.

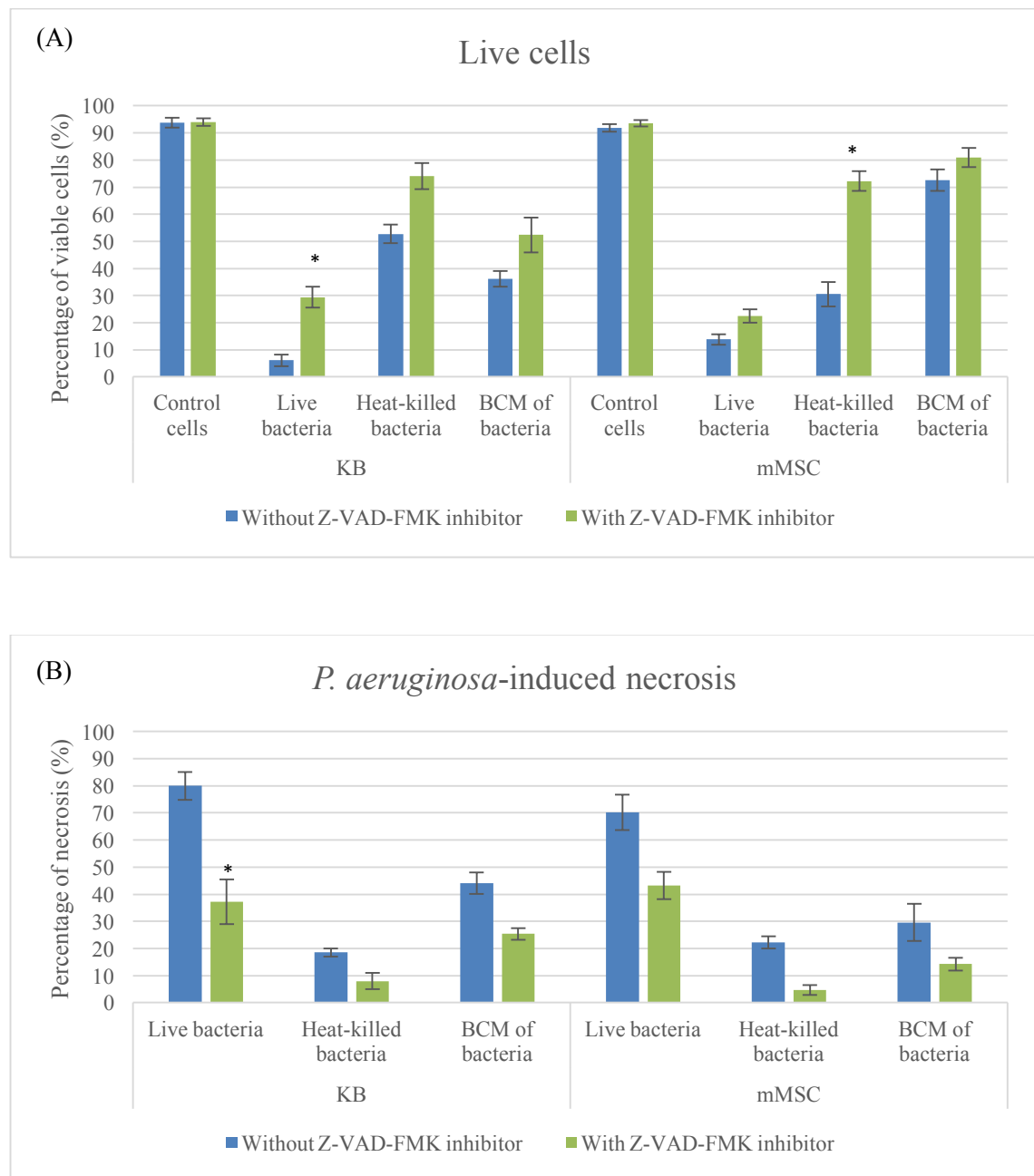


Figure 4.6 Evaluation of caspase pathway role in cell death induced by *P. aeruginosa* on KB epithelial cell line and mMSC cells.

Both cell types were infected with live or heat-killed bacteria (10 CFU/cell) or BCM of *P. aeruginosa* for 24 h. (A) Quantification of viable cells following exposure to live or heat-killed bacteria (10 CFU/cell) or BCM of *P. aeruginosa* for 24 h with and without Z-VAD-FMK inhibitor assessed by annexin V-FITC and Zombie NIR by flow cytometer (- annexin V-FITC / - Zombie NIR). (B) Quantification of necrotic cells following exposure to live or heat-killed bacteria (10 CFU/cell) or BCM of *P. aeruginosa* for 24 h with and without Z-VAD-FMK inhibitor assessed by measuring LDH release into the supernatant using a colorimetric assay. Viable cells were increased as a result of apoptotic cells reduction by inhibit caspase pathway. Necrotic cells were slightly decreased when caspase pathway inhibited. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

4.3.4 Role of caspase pathway in CM of *Acanthamoeba*-induced cell death

Caspase cascade role in CM of *Acanthamoeba*-induced cell death was determined by fluorescence and colorimetric techniques, and using Z-VAD-FMK inhibitor. KB epithelial cell line and mMSC cells were infected by CM of *Acanthamoeba* for 24 h in the presence and absence of caspase inhibitor. Percentages of necrotic cells were measured by detecting the amount of LDH released from necrotic cells by colorimetric assays, while viable and apoptotic cells were determined by flow cytometry after staining with annexin V-FITC and Zombie NIR.

Apoptosis induced by *Acanthamoeba* CM in KB epithelial cells was extremely high, at 96 %, and that was slightly reduced by using caspase inhibitor to be 83 % (Figure 4.7). Necrotic KB epithelial cells decreased from 62 ± 8 % to 40 ± 3 %, while the percentage of viable cells was increased from 10 ± 2 % to 22 ± 4 % (Figure 4.7 and Figure 4.8a,b). mMSC cells treated with caspase inhibitor showed a lower percentage of apoptotic cells (38 %), while those untreated with the inhibitor showed 96 % apoptotic cells (Figure 4.7). The percentage of viable cells increased from 8 ± 3 % to 57 ± 8 %; while necrosis decreased from 61 ± 5 % to 21 ± 7 % (Figure 4.7 and Figure 4.8a,b).

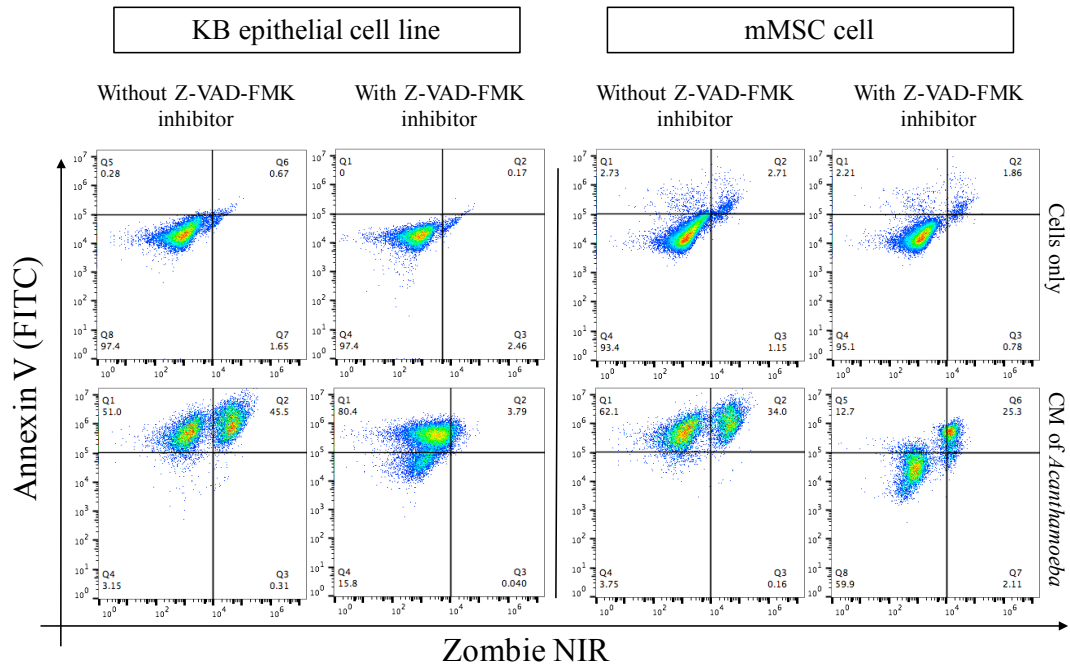


Figure 4.7 Evaluation of caspase pathway role in apoptosis induced by CM of *Acanthamoeba* on KB epithelial cell line and mMSC cells.

Both cell types were treated with *Acanthamoeba* CM for 24 h. Cells were stained with annexin V-FITC and Zombie NIR and analysed by flow cytometry. Cells double positive for both annexin V-FITC and Zombie NIR were dead cells, while cells with positive annexin V-FITC and negative Zombie NIR were apoptotic cells. Live cells were those cells with low Zombie NIR and negative annexin V-FITC. Controls (cells only) showed high percentage of viable cells with very low apoptotic and dead cells. Cells treated with CM of *Acanthamoeba* showed a very low number of viable cells with increased numbers of apoptotic and dead cells compared with untreated cells. Live cells increased after using Z-VAD-FMK inhibitor in both host cells types. Shown is a representative of four experiments.

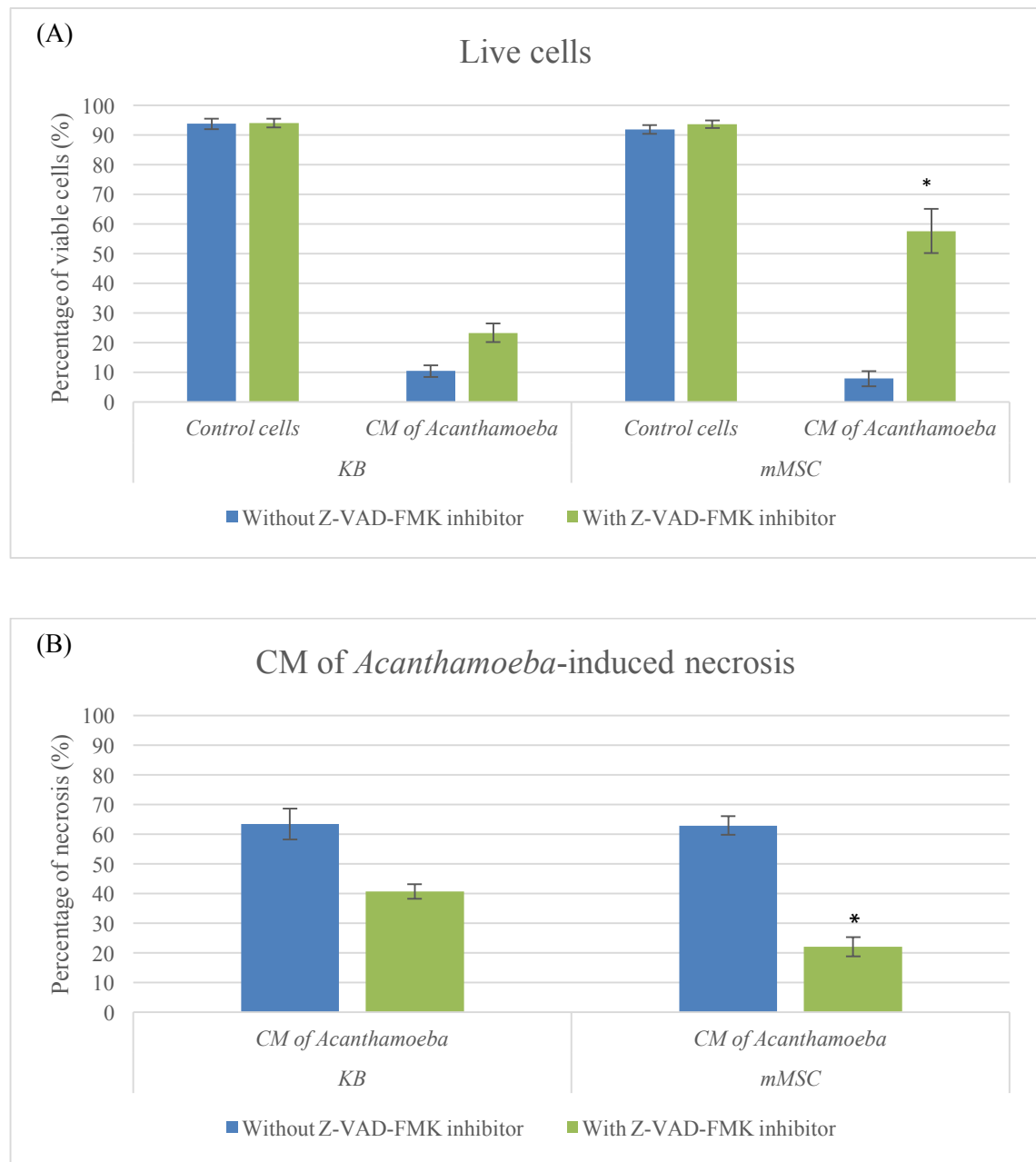


Figure 4.8 Evaluation of caspase pathway role in cell death induced by CM of *Acanthamoeba* on KB epithelial cell line and mMSC cells.

Both cell types were treated with *Acanthamoeba* CM for 24 h. (A) Quantification of viable cells following exposure *Acanthamoeba* CM for 24 h with and without Z-VAD-FMK inhibitor assessed by annexin V-FITC and Zombie NIR by flow cytometer (- annexin V-FITC/ - Zombie NIR). (B) Quantification of necrotic cells following exposure to *Acanthamoeba* CM for 24 h with and without Z-VAD-FMK inhibitor assessed by measuring LDH release into the supernatant using a colorimetric assay. Viable cells were increased as a result of apoptotic cells reduction by inhibiting the caspase pathway. Necrotic cells were slightly decreased when the caspase pathway was inhibited. Results are the mean of three independent experiments performed in duplicate. Error bars represent standard error. Statistical analysis: two-tailed t-test (* = $P < 0.05$).

4.4 Discussion

Wound healing includes a sequence of specific phases which enhance cells in tissue remodelling and increase the population of the cells to repair the injured area by rebuilding new matrices then maturing the wound. Different cells are recruited to wounds to complete these stages, including MSCs. When this process is completed, unwanted cells in the wound are eliminated via a fundamental biological process which provides tissue homeostasis, which is cell death. Two forms of cell death are involved in wound healing and wound infections: apoptosis and necrosis. Cell death forms are involved in wound infection as an immune response to infection to eliminate infected cells and to clear the wound by macrophages (Serra *et al.*, 2015). In addition, it is well known that microorganisms have a role in cell death. However, each microorganism has different virulence factors, including contact-dependent factors (e.g. FnBPs, pili and flagella) and contact-independent factors (e.g. α -toxin, ExoS and Ecto-ATPases) (Worgall *et al.*, 2002; Haslinger-Löffler *et al.*, 2005; Khan, 2009). This chapter studied the overall effect of these factors in inducing cell death in skin epithelial cells and mesenchymal stem cells (MSC), and also the role of caspase cascade in cell death induced by live or heat-killed bacteria or CM of both bacteria and *Acanthamoeba*.

The present study showed that both primary culture of mMSC and KB epithelial cell line are sensitive to MRSA-, *P. aeruginosa*- and *Acanthamoeba*-induced cell death. In addition, the caspase cascade has a role in both forms of cell death (apoptosis and necrosis). Cell death of KB epithelial cell line induced by *P. aeruginosa* was higher than that induced by MRSA at early times post-infection. Apoptotic and necrotic responses of mMSC and KB epithelial cells to MRSA and *P. aeruginosa* were dependent on live bacteria and BCM, as they were higher than heat-killed bacteria. Inhibition of caspase reduced the degree of host cell death. Importantly, CM of

Acanthamoeba-induced cell death was significantly decreased in mMSC when caspase inhibitor was used, presenting a higher percentage of viable cells compared with KB epithelial cells. Together, these observations support the concept that toxins, enzymes and proteases produced by pathogens may have a major role in delaying wound healing, in part, by inducing cytotoxic effects on cells. Although caspase-3 has a regulated role in apoptosis, while caspase-8 has a role in both apoptosis and necrosis (Hetz *et al.*, 2002; Haslinger-Löffler *et al.*, 2005; McIlwain *et al.*, 2013), our findings showed the ability of caspase-3 inhibitor (Z-VAD-FMK) to modify the necrotic effect on cells. In necrosis, ROS are produced as a result of external or internal signals, such as bacterial toxins, while apoptotic bodies result from apoptosis induced by an external agent (Behar *et al.*, 2010; Kim *et al.*, 2010). *In vivo*, macrophages phagocytose apoptotic bodies with intracellular bacteria. However, *in vitro* this process is completely different as no phagocytosis happened, thus, apoptotic bodies would be burst and necrosis will take place as a result of multiplication of the intracellular viable bacteria or via prolonged exposure times to lytic toxins and enzymes produced by microorganisms. As a result, inhibiting apoptosis could indirectly inhibit necrosis.

Several studies indicated that *S. aureus* were able to induce cell death in different types of cells such as epithelial cells and endothelial cells, using different virulence factors e.g. haemolysin α -toxin, TSST-1 and FnBPs (Kahl *et al.*, 2000; da Silva *et al.*, 2004; Essmann *et al.*, 2003; Mateo *et al.*, 2007). *S. aureus* use their factors in order to invade and colonise host cells (Peacock *et al.*, 2002). Furthermore, *P. aeruginosa* have the ability to induce cell death in different cell types, such as lung epithelial cell line, as demonstrated by several studies (Worgall *et al.*, 2002; Losa *et al.*, 2014; Wood *et al.*, 2015). Different virulence factors produced by *P. aeruginosa* have been proved to be cell death inducers. T3SS effector proteins ExoT, ExoS and ExoU have an important

role in cell death induction. In addition, pyocyanin factor produced by *P. aeruginosa* within the direct interaction of *P. aeruginosa* with host cells might have the ability to induce cell death in epithelial cells (Losa *et al.*, 2014; Broquet and Asehnoune, 2015).

Different studies have proved the ability of *Acanthamoeba* trophozoites and their lysate to cause cell death (Zheng *et al.*, 2004; Chusattayanond *et al.*, 2010). *Acanthamoeba* trophozoites produce Ecto-ATPases, which hydrolyse the extracellular ATP to ADP. ADP will be released as a result of *Acanthamoeba* binding with host cells causing caspase-3 activation and then apoptosis (Khan, 2009). However, the role of *Acanthamoeba* in wound infections is not very well understood due to the uncommon incidence of such disease. As mentioned in the introduction, one case study has been published showing the presence of *Acanthamoeba* in a lung transplant patient who died a few days later from developing AGE. This is the first study to isolate *Acanthamoeba* from clinical wound samples from healthy individuals. This will be explained in details in the next chapter when 140 samples were checked for the presence of *Acanthamoeba* in Colchester.

Chapter Five

5

Investigation of the presence of *Acanthamoeba*
in wound samples

Chapter 5 : Investigation of the presence of *Acanthamoeba* in wound samples

5.1 Introduction

FLA are widely distributed in the environment. They can be isolated from soil, water and other environmental sources. A few species, including *Acanthamoeba* spp., were found as causative agents for fatal infections of the CNS called, in the case of *Acanthamoeba*, AGE, especially in immunocompromised patients. However, cutaneous diseases such as non-healing skin lesions in the absence of CNS involvement are increasingly recognized (Torno *et al.*, 2000). One of the entry routes for *Acanthamoeba* is through skin lesions, then via bloodstream to the BBB. The nature of wounds provide an open source for microorganisms to enter and cause an infection. A case report has shown the presence of *Acanthamoeba* trophozoites and cysts in punch biopsy of skin nodule from a 62-year-old male who had history of lung transplant (D'Auria *et al.*, 2012). However, till now, there has not been a single study to isolate *Acanthamoeba* from wound samples published.

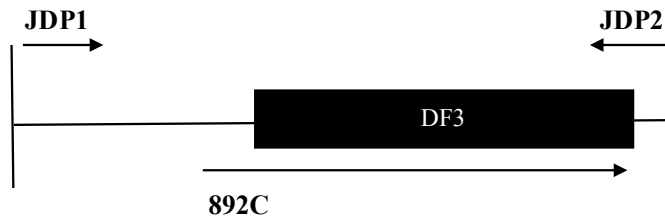
Not only immunocompromised patients at risk of *Acanthamoeba*, healthy individuals whom wearing contact lens are at the risk as well of gaining *Acanthamoeba* keratitis (AK). The number of diagnosed cases of AK has increased dramatically within the last two decades. The relation between contact lens wearer and AK has been well established, and spotlighted on contact lens and their containers. The wide distribution of *Acanthamoeba* in the environment gave them high chance to contaminate contact lens and containers, which may be resulting AK (Gomes *et al.*, 2016). Other recent study had detected *Acanthamoeba* in ocular surface of healthy individuals. All those patients were non-contact lens wearer (Rocha-Cabrera *et al.*, 2015).

Genus of *Acanthamoeba* comprises about 20 genotypes. This classification based on sequences of Diagnostic Fragment 3 (DF3) of 18S rDNA of the genus *Acanthamoeba* (Siddiqui and Khan, 2012; Fuerst, 2014; Corsaro *et al.*, 2015). Before the year 2000, genetically cloned materials were used to obtain most *Acanthamoeba* sequences. In the same time, a number of PCR primers were tested to produce partial products of 18S rRNA gene to diagnose infections involving *Acanthamoeba*. In 2001, a series of PCR primers were designed and developed by a group from Ohio State University (OSU). Some of those primers become widely used to identify and sequence clinical and environmental isolates of *Acanthamoeba* (Schroeder *et al.*, 2001). This pair of PCR primers used to amplify a region of *Acanthamoeba*-specific amplicon S1 (ASA.S1) with size approximately 400-460 base pairs (bp). The ASA.S1 amplicon sequences showed variation between different strains within 18S rDNA and then was used for genotyping (Stothard *et al.*, 1998). There is another PCR primers pair which was designed to amplify a large region that was called genotype-specific amplicon B1 (GTSA.B1). In this amplicon, three variable diagnostic fragments (DF1, DF2 and DF3) can be sequenced. While only DF3 can be found in ASA.S1 amplicon. A specific primer called (892C) can be used to sequence DF3 within ASA.S1 amplicon giving approximately 200-250 bp in size that includes most of the highly variable portions of the ASA.S1 region. Sequences of both ASA.S1 and DF3 regions from 18S rDNA can be used for the phylogenetic signal (Figure 5.1.). The partial sequences can be misleading with phylogenetic relationships especially in the less frequently reported *Acanthamoeba* isolates. However, phylogenetic analysis of *Acanthamoeba* which rely on the almost complete sequences show very accurate results (Schroeder *et al.*, 2001).

Herein the presence of *Acanthamoeba* has been investigated, within clinical wound swabs which had already been collected for routine bacterial investigation in Colchester

General Hospital, by culturing on 3 % Non-Nutrient Agar (NNA). Then the variable specific region ASA.S1 and sequence DF3 were amplified to identify the genotype of positive samples. Thus the phylogenetic relationships between positive samples could be compared with other different genotypes from the database.

(A) Sequenced region of ASA.S1



(B) Sequenced region of GTSA.B1



Figure 5.1 Sequenced regions, Variable regions, and primers used for PCR and sequencing of ASA.S1 and GTSA.B1.

(A) Shows ASA.S1 amplimer, JDP1-JDP2 primers pair and primer 892C, primers indicated by the arrows. The black box represents DF3. (B) Black boxes represent the diagnostic regions (DF1, DF2 and D3) within GTSA.B1. The locations and directions of the amplification primers and the sequencing primers are indicated by arrows.

5.2 Materials and Methods

5.2.1 Ethical approval and forms

Ethical approval was required for this study. Application for ethical approval had three phases. Firstly, the ethical approval application was reviewed by research and enterprise office at the University of Essex to obtain a sponsor confirmation letter. Then, the ethical approval application was submitted online through Integrated Research Application System (IRAS) website [<https://www.myresearchproject.org.uk>] to NHS Research Ethics (London-Chelsea Committee) for proportionate review, where a favourable opinion subject was issued (reference no. 14/LO/1538). Finally, NHS Research and Development (NHS R&D) approval form and Site-Specific Information (SSI) form were completed through the IRAS website and sent with all supporting documents to Colchester General Hospital NHS Trust's Research and Development office for approval. The NHS R&D approval was issued (reference no. 2014/039).

5.2.2 Clinical samples

Clinical swabs from different sites of wounds and ulcers from patients at Colchester General Hospital were collected by the microbiology unit at Colchester General Hospital for routine bacterial investigation; these were then tested for the presence of *Acanthamoeba* at the medical microbiology laboratory, School of Biological Sciences, University of Essex, Colchester. A total of 140 wound swabs were tested. To determine the presence of *Acanthamoeba*, specimens were inoculated on to 3 % NNA (Oxoid) plates seeded with *E. coli* K12. Plates were incubated at 30 °C and observed daily for the presence of amoebae. *Acanthamoeba* were monoxenically subcultured onto other NNA plates and then axenically cultivated in PYG medium at 30 °C as described previously (Khan and Paget, 2002).

5.2.3 Xenic culture of *Acanthamoeba*

To determine the presence of *Acanthamoeba*, specimens were cultured by monoxenic growth on NNA plates seeded with a lawn of *E. coli* (NNA-*E. coli*) (Page, 1988). The NNA medium was comprised of 1.5 % plain agar (Agar No. 1, Oxoid, U.K.) in Page's Amoeba saline (PAS) [Sodium Phosphate 0.0142 g, Potassium Phosphate 0.0136 g, Sodium Chloride 0.012 g, Magnesium Sulfate 0.0004 g, Calcium Chloride 0.0004 g, and Agar 15 g] in 1 L of deionized water] and the final pH was 7.0 ± 0.2 . The agar mix was autoclaved at 121 °C for 15 min, and allowed to cool to 50 °C before being poured into Petri dishes, and left to gel overnight at 37 °C.

Once set, the agar surface was seeded with 5 mL of a dense suspension of *E. coli* K12 (ATCC 33876) (see 5.2.4.). Approximately 5 mL of late log phase cultures of *E. coli* were poured onto NNA plates and left for 5 min, after which excess culture fluid was removed and plates were left to dry before their inoculation with clinical specimens.

For routine maintenance of *Acanthamoeba*, plates were cultured in air incubator at 30 °C. All plates were observed daily for the presence of amoebae. To isolate and clone single cysts from a mixed culture, cultures were refreshed weekly by excising a piece region of agar from the leading edge of the plaque, containing trophozoites, and placing it face down onto the centre of a fresh NNA-*E. coli* plate and incubated as described above.

5.2.4 Preparation of *E. coli* food source stock

To make the stock suspension, *E. coli* was streaked on to a CLED agar plate using a sterile disposable loop. Once inoculated, the CLED plates were incubated overnight at 37 °C allowing colonies to form.

Distinct single colonies were picked with a loop, and transferred to a sterile glass bottle with 100 mL of LB broth, and propagated overnight at 37 °C in air incubator. The

suspension of *E. coli* was pelleted by centrifugation at 2,000 x g for 30 min, and the supernatant was discarded and the pellet washed by resuspension in PAS. This step was repeated three times, with the final pellet being resuspended in 50 mL of PAS. The stock of *E. coli* to be used as a food source can be stored at 4 °C for up to two weeks.

5.2.5 Axenic culture of *Acanthamoeba*

Where possible, strain was adapted to axenic growth in PYG medium supplemented with Penicillin/Streptomycin (to a final concentration of 100 U/mL and 0.1 mg/mL, respectively) (Hughes and Kilvington, 2001). The complete medium can be stored at 4 °C, and used within one month.

Before axenic culture could be obtained, superfluous bacteria surrounding the *Acanthamoeba* were removed by treating cysts with 2 % (v/v) hydrochloric acid (HCl) for 24 h (Kilvington and White, 1994). All traces of HCl were then removed by washing the cysts with sterile deionised water and pelleting with centrifugation at 3,000 x g for 5 min, this wash step was repeated three times. Washed cysts were inoculated into flat-sided Nunclon™ Surface tissue culture 6 well-plate (Fisher Scientific UK) containing 3 mL of culture media, and incubated in air at 30 °C. Under these conditions, excystation should occur and any emergent trophozoites can adapt and replicate in the media. Then, axenic strain was successfully maintained in 25 cm² tissue culture flasks after six months of the main swab was collected (Nunc- Fisher Scientific UK).

5.2.6 DNA extraction

The growing amoebae were harvested from culture plates and collected in centrifuge tubes. Then, amoeba were washed twice with 100 µl of PBS 1X. The DNA was extracted by using the QIAamp DNA Micro Kit (QIAGEN Ltd., Crawley, UK) following manufacturer's guidelines. Briefly, approximately 10³ amoeba (counted using a haemocytometer) were suspended with 180 µl of Tissue Lysis Buffer (ATL). Tubes

were incubated into the water bath at 56 °C for 4-6 h. Then, mixed solution of 200 µl of Buffer AL with 1 µl Carrier RNA were added to each tube; and they were vortexed until the pellet completely dissolved. About 200 µl Ethanol (95-100 %) were added to each sample and mixed well. The sample tubes were lifted for 5 min at room temperature. Samples were transferred to QIAamp MinElute columns and centrifuged for 1 min at 8000 x g. The collection tubes were discarded and replaced with new collection tubes. The sample tubes were washed by adding 500 µl of the first washing buffer (AW1) and centrifuged for 1 min at 8000 x g. The collection tubes were discarded and replaced with new collection tubes. Samples were washed again by adding 500 µl of second washing buffer (AW2) and centrifuged for 1 min 8000 x g. Next, the collection tubes were discarded and replaced with new tubes, and centrifuged for 3 min 14000 x g, to dry the membrane. The DNA sample extracts were collected in 1.5 centrifuge tubes by adding 50 µl of elution buffer (AE) in the centre of the membrane and left on the bench for 1-5 min at room temperature, then centrifuged for 1min 14000 x g. The DNA extractions were loaded onto 1 % agarose gel, to detect the DNA extraction.

5.2.7 PCR methods analysis

A standard PCR method was used for the detection of *Acanthamoeba*. PCR reactions were performed following the cycling structure and conditions described by Schroeder et al 2001. Sequencing forward and reverse primers JDP1 (5'-GGCCCAGATCGTTTACCGTGAA) and JDP2 (5'-TCTCACAAGCTGCTAGGGGAGTCA) which amplify a fragment of approximately 500 bp of the ASA.S1 amplicon that included DF3 was used to amplify amplicons of the isolates for genotyping purposes as described previously (Schroeder *et al.*, 2001). PCR was performed in a volume of 50 µl containing 1.25 U Taq polymerase (Thermo Scientific), 1–10 ng DNA, 200 µM dNTPs, 4 mM MgCl₂ and 0.5 µM primer. The PCR

cycle profile was as follows: initial denaturation at 95 °C for 5 min, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 30 sec, with a final extension at 72 °C for 7 min. As a negative control for amplification (non-target), reaction mixtures containing only reagents and sterile distilled water were prepared and included simultaneously with the unknowns in each PCR run. The total PCR product was visualized on Safe view DNA-stained 1 % agarose gels (Fisher). After detecting the PCR product in the gel, the DNA-containing fragment was cut from the agarose gel with a clean, sharp scalpel and the size of the gel slice minimized by removing extra agarose, and put in 1.5 mL centrifuge tubes to purify.

5.2.8 Purification of PCR product

The PCR product was purified with the QIAquick Gel Extraction Kit (QIAGEN Ltd., Crawley, UK) following manufacturer's guidelines. Briefly, the gel slice containing DNA fragments were transferred to colourless tube containing 200 µl of QG buffer. Next, tubes were incubated at 50 °C in water bath until the gel slice had completely dissolved and the color changed to be yellow. Then, about 200 µl of isopropanol was added to each sample and mixed. Samples were transferred to QIAquick spin column and centrifuged for 1 min at 13000 x g. Then, about 500 µl of QG buffer were added to the column after the content of the collection tubes were discarded and centrifuged for 1 min at 13000 x g. Next, samples were washed once with 750 µl of PE buffer and centrifuged for 1 min at 13000 x g. Then, the washings were discarded and the QIAquick columns were centrifuged for an additional 1 min at ~13,000 g to dry the membrane. The QIAquick columns were then placed into a clean 1.5-mL microfuge tube to elute the DNA by adding 30 µl of EB buffer to the center of the QIAquick column. All the columns were left to stand for 1 min, and then centrifuged for 1 min at 14000 x g.

5.2.9 18s rDNA gene sequencing

The DF3 fragment of positive control and positive samples were commercially sequenced using Sanger Sequencing services (SourceBioscience; Birmingham, UK). One way 892C primer was used to obtained DF3 sequences.

5.2.10 Phylogenetic analysis and Blast

Genotype assignment was based on sequence analysis of the 18S rDNA gene including the DF3 fragment. Multiple sequences of the different *Acanthamoeba* genotypes of the same region were performed by using ClustalW via MEGA (version 6.06). Sequences were identified via a Basic Alignment Search Tool (BLAST), by using nucleotide blast to locate the highly similar sequences for comparison. Nucleotide sequences of the homologous gene fragments from all genotypes were retrieved from GenBank (see table 5.1 and table 5.2 for accession numbers). Phylogenetic analysis based on the sequences obtained was performed using MEGA. Distance-based analyses were conducted using the maximum composite likelihood method, and trees were constructed using a Neighbour-Joining algorithm. Bootstrap proportions were calculated by analysing 1000 replicates (Cabello-Vílchez *et al.*, 2014b). Maximum composite likelihood method was selected to estimate evaluation distances between DNA sequences of *Acanthamoeba* genotypes with their differences by a bootstrap approach. The advantage of using this method is to reduce errors, as a single set of parameters estimated from all sequence pairs is applied to each distance estimation. When distances are estimated with lower errors, distance-based methods for inferring phylogenies are expected to be more accurate. Therefore, this method will be giving high bootstrap values for the estimated phylogenetic tree.

Genotype	Isolate	Genbank (accession #)
T1	<i>A. castellanii</i> [ATCC 50494]	(U07400)
T2	<i>A. palestinensis</i> [ATCC 30870]	(U07411)
T3	<i>A. griffini</i> [ATCC 30731]	(U07412)
T4	<i>A. castellanii</i> [ATCC 30011]	(U07413)
T5	<i>A. lenticulata</i> [ATCC 50428]	(U94739)
T6	<i>A. palestinensis</i> [ATCC 50708]	(AF019063)
T7	<i>A. astronyxis</i> [ATCC 30137]	(AF019064)
T8	<i>A. tubiashi</i> [ATCC 30867]	(AF019065)
T9	<i>A. comandoni</i> [ATCC 30135]	(AF019066)
T10	<i>A. culbertsoni</i> [ATCC 30171]	(AF019067)
T11	<i>A. hatchetti</i> BH-2 [ATCC 30730]	(AF019068)
T12	<i>A. healyi</i> [ATCC 30866]	(AF019070)
T13	<i>A. spp.</i> UWC9 [ATCC PRA-3]	(AF132134)
T14	<i>A. spp.</i> PN13	(AF333609)
T15	<i>A. jacobsi</i> [ATCC 30732]	(AY262360)
T16	<i>A. spp.</i> U/HC1	(AY026245)
T17	<i>A. spp.</i> Ac E1a	(GU808277)
T18	<i>A. spp.</i> CDC:V621	(KC822461)
T19	<i>A. spp.</i> USP-AWW-A68	(KJ413084)
T20	<i>A. spp.</i> OSU 04-020	(DQ451161)

Table 5.1 Different *Acanthamoeba* genotypes which were used to obtain phylogenetic tree.

Genotype	Isolate name	Genbank (accession #)
T4A	<i>A. castellanii</i> [ATCC 30011]	(U07413)
T4B	<i>A. castellanii</i> Ma [ATCC 50370]	(U07414)
T4C	<i>A. spp.</i> Fernandez [ATCC 50369]	(U07409)
T4D	<i>A. rhysodes</i> Singh [ATCC 30973]	(AY351644)
T4E	<i>A. polyphaga</i> Page-23 [ATCC 30871]	(AF019061)
T4F	<i>A. triangularis</i> SH621 [ATCC 50254]	(AF346662)
T4-Neff	<i>A. castellanii</i> Neff [ATCC 30010]	(U07416)

Table 5.2 Different subtypes isolate of *Acanthamoeba* belonging to T4 genotype which were used to obtain phylogenetic tree.

5.3 Results

One out of 140 wound swab samples was positive for *Acanthamoeba* spp., beside positive control *A. castellanii* (T4 genotype). The positive sample was isolated from an 85 year-old female with leg wound. Her first episode was treated with flucloxacillin however, recovery was followed by an acute episode which was treated with trimethoprim. Both positive sample and positive control were cloned to obtain pure isolations for axenification and further molecular investigation (Figure 5.2.). DNA extracted from those samples, and ASA.S1 region were successfully amplified (Figure 5.3.). DF3 region in both sample and control were sequenced and aligned alongside the other *Acanthamoeba* species obtained from the database and shown in (table 5.1.) (Figure 5.4.). The alignment results show the differences in the variable region, however, both sample and control showed some similarity with the T4 genotype that was used in the alignment (Figure 5.5.). To find the relationships between these strains a phylogenetic tree was obtained (Figure 5.6.). Further alignment was done for both sample and control with 7 different subtypes of *Acanthamoeba* T4 genotype. Both of them belong to T4A subtype genotype (Figure 5.7.). After employing nucleotide BLAST, it became clear the positive sample was identified as *A. castellanii* (Figure 5.8.).

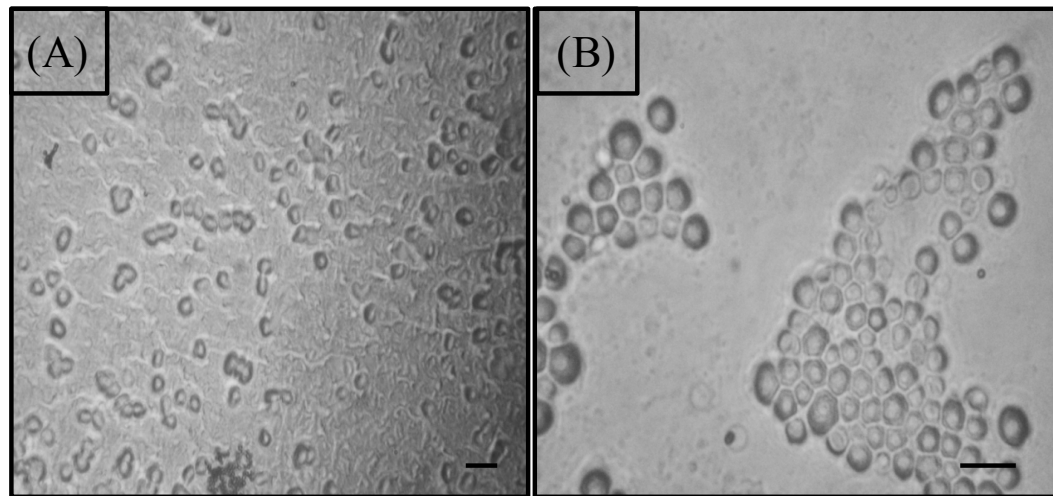


Figure 5.2 Presence of *Acanthamoeba* spp. in the only positive wound samples.

Image (A) show *Acanthamoeba* trophozoites and their feed tracks on NNA; and image (B) show clumped *Acanthamoeba* cysts on NNA. X40 objective (bars = 50 μm).

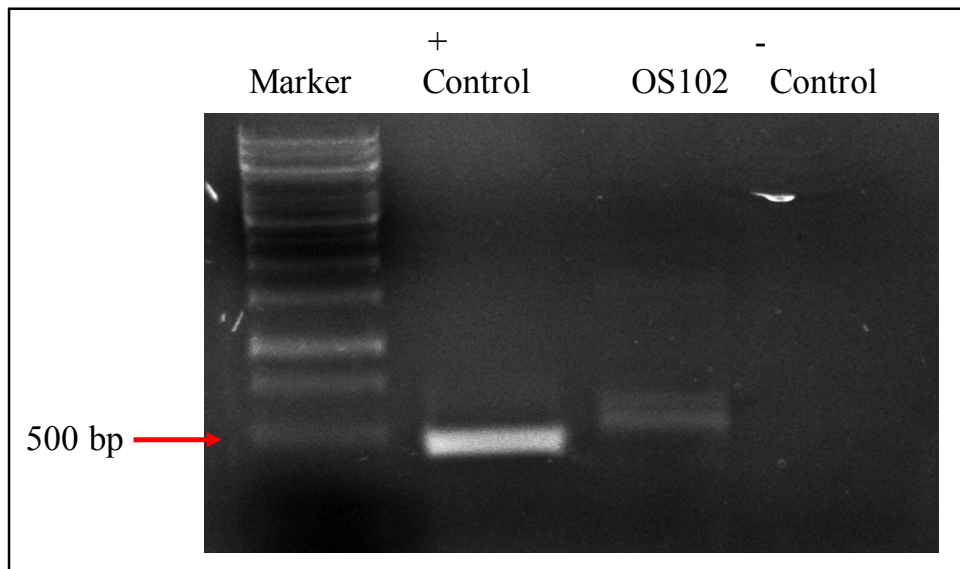


Figure 5.3 ASA.S1 amplicon was present in both sample and positive control with size approximately 500 bp.

Lane 1: 1kb DNA ladder (Thermo scientific #SM0311), Lane 2: Positive control *A. castellanii*, Lane 3: sample (OS102), and Lane4: Negative control (water).

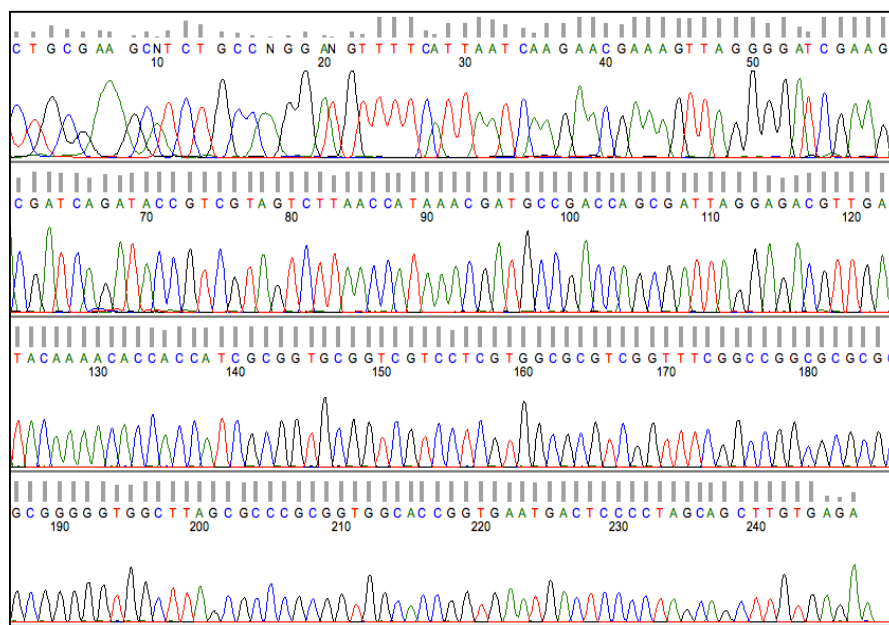


Figure 5.4 Sequence of DF3 fragment of the positive wound swab sample (OS102).

One single primer (892C) was used to obtain DF3 sequences.

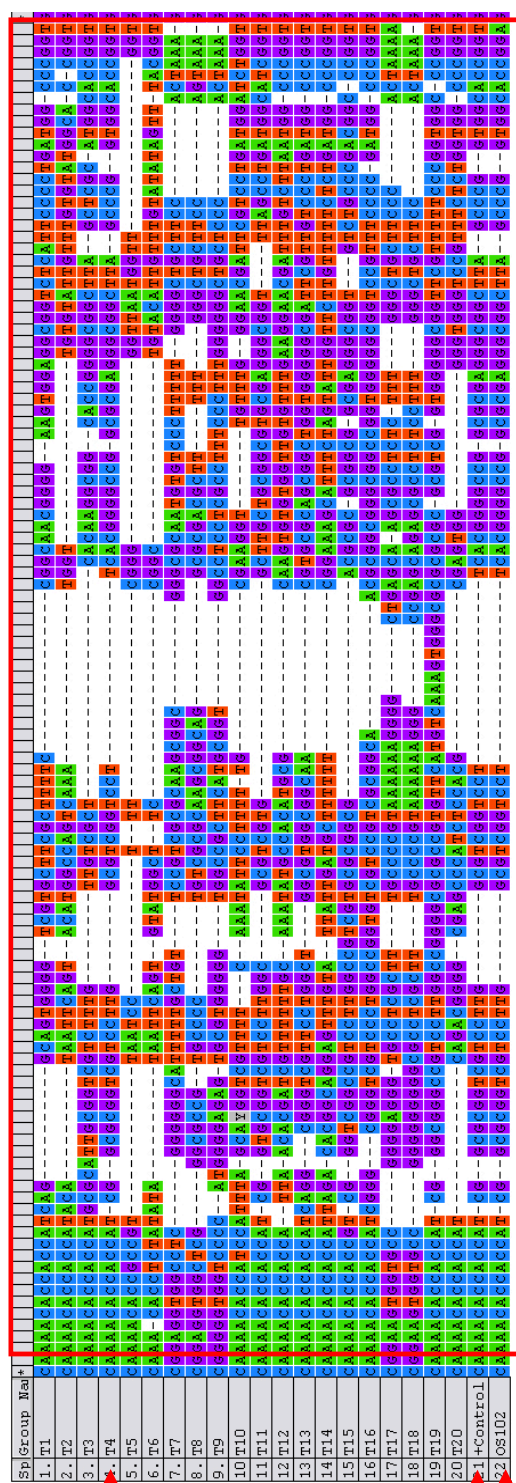


Figure 5.5 Primary sequence alignment of a variable region of DF3 of 18S rDNA.

Primary sequence alignment of a variable region of DF3 of 18S rDNA of sample OS102 beside positive control and 20 genotypes of *Acanthamoeba* (T1-T20) by MEGA 6.06. The alignment shown (red box) is a subset of DF3 that contains the highly variable and informative section of this fragment. Arrows refer to T4 genotype from the database (4), positive control which is *A. casteallanii* (21), and positive sample (OS102) (22).

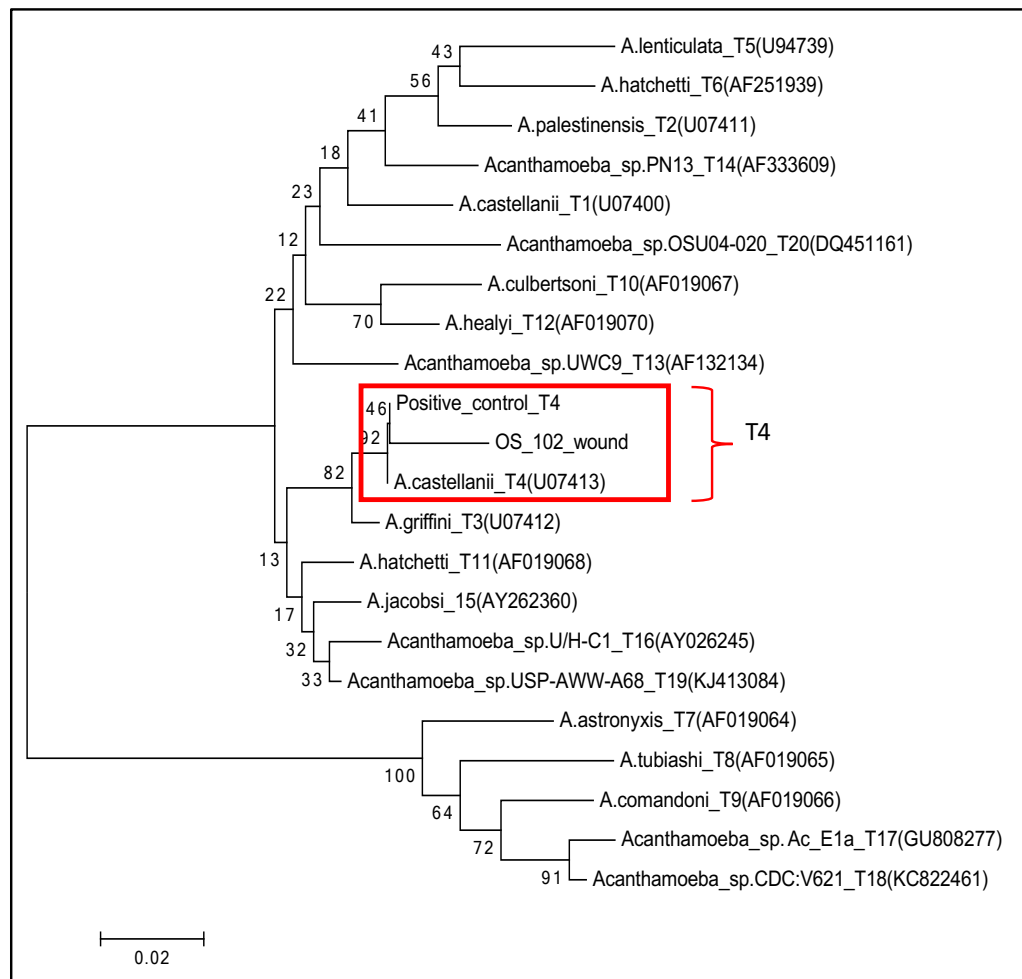
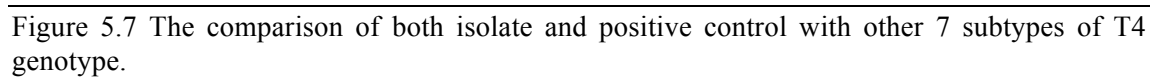


Figure 5.6 18S rDNA DF3 phylogenetic neighbour-joining tree.

18S rDNA DF3 phylogenetic neighbour-joining tree obtained by using two-parameter distance algorithm, produced in MEGA 6.06. The isolate and positive control obtained in this study are identified in the tree (red box). Bootstrap value is 46%.



Go to alignments		Description	Max score	Total score	Query cover	E value	Ident	Accession
<input checked="" type="checkbox"/>		Acanthamoeba castellanii strain ATCC 30234 18S ribosomal RNA gene, partial sequence	353	702	96%	6e-94	95%	KP221679.1
<input type="checkbox"/>		Acanthamoeba sp. Tib142 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	KF928956.1
<input type="checkbox"/>		Acanthamoeba sp. Tib22 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	KF928951.1
<input type="checkbox"/>		Acanthamoeba castellanii strain ATCC 30011 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	KF318462.1
<input type="checkbox"/>		Acanthamoeba genotype T4 voucher OSU10-002 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	JX423602.1
<input type="checkbox"/>		Acanthamoeba genotype T4 voucher OSU10-001 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	JX423601.1
<input type="checkbox"/>		Acanthamoeba genotype T4 voucher OSU06-059 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	JX423584.1
<input type="checkbox"/>		Acanthamoeba genotype T4 voucher OSU06-051 18S ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	JX423580.1
<input type="checkbox"/>		Acanthamoeba genotype T4 isolate OSU#09-007 small subunit ribosomal RNA gene, partial sequence	353	353	96%	6e-94	95%	JQ669659.1

Figure 5.8 Sequence of DF3.

Sequence of DF3 was identified via a basic alignment search tool (BLAST, <https://blast.ncbi.nlm.nih.gov>), by using nucleotide blast to locate the highly similar sequence for comparison. The isolate from wound was identified as *A. castellanii*.

5.4 Discussion

This is the first study to look at the genomic characterisation of *Acanthamoeba* strains from wounds in the worldwide, and the first report of *Acanthamoeba* in Colchester and whole Essex. The world-wide distribution of *Acanthamoeba* gives them the opportunity to be one of the microorganism which could be colonise an open wound. *Acanthamoeba* species were isolated from both clinical and environmental sources, including soil, waste dumps, cooling towers of air conditioning systems, humidifiers, aquaria, dialysis machines, dental equipment, as well as bottled, tap, and sea waters (Siddiqui and Khan, 2012). Moreover, *Acanthamoeba* were isolated from the nasal samples of healthy people indicating that subclinical infections might be common (Cabello-Vílchez *et al.*, 2014b).

There are two main clinical infections caused by *Acanthamoeba*: AK in healthy people who wear contact lenses and AGE in immunosuppressed people. One of *Acanthamoeba* entry routes for CNS is through a skin lesion (cutaneous acanthamoebiasis) (Torno *et al.*, 2000). The importance of AGE in the literature has been emphasised, especially in immunocompromised patients, such as AIDS patients and patients who have organ transplant (Marciano-Cabral and Cabral, 2003). In 2012, D'Auria *et al.* reported a 66-year-old male who had a double lung transplant, after which he was diagnosed with cutaneous acanthamoebiasis which was then followed by AGE. In the literature, about 20 cases of transplant patients got *Acanthamoeba* spp. infections, and only 8 of them presented with skin lesions. Those lesions were nodules, ulcers and cellulitis (D'Auria *et al.*, 2012). However, there is not a single study to report isolation of *Acanthamoeba* from a wound in an immunocompetent person.

In the present study, swabs were obtained from different wounds, including abscess, ulcer, and other skin wounds. One out of 140 samples was positive, and that was from a

right-leg-wound. The number of positive samples in this study is very low compared with other studies. The recommended samples for amoeba clinical isolations are cerebrospinal fluid (CSF), biopsy, or tissue specimens (Centers for Disease Control and Prevention, 2014). In a very recent study, about 28 % of the total samples from nasal swabs were positive for *Acanthamoeba* (Cabello-Vílchez *et al.*, 2014b). However, some other previous studies had shown very low percentages of positive samples (Cerva *et al.*, 1973; Michel *et al.*, 1982; Badenoch *et al.*, 1988). In another study which tried to isolate *Acanthamoeba* spp. from conjunctiva swabs, no single sample out of 286 samples was positive for *Acanthamoeba* (Anisah *et al.*, 2005). Therefore, swabs may not be the best sampling method for clinical *Acanthamoeba* isolations compared with biopsy or tissue specimens.

Based on the inconsistencies in classifying *Acanthamoeba* spp. according to their morphology, PCR was used to identify *Acanthamoeba* spp. based on the sequence of ASA.S1amplimer (Schuster and Visvesvara, 2004). Genus *Acanthamoeba* is divided into 20 genotypes based on the sequences of 18s rDNA (Corsaro *et al.*, 2015). Nevertheless, only some of these genotypes are associated with human infections, other genotypes have been found only in the environment; however, T4 genotype was found in both clinical and environmental samples (Booton *et al.*, 2005; Khan, 2006; Ledee *et al.*, 2009; Magnet *et al.*, 2012). Moreover, the T4 genotype has been identified as a main one associated with both AK and AGE. More than 90 % of AK cases are caused by T4 genotype; and that genotype has been linked with AGE and cutaneous infections (Siddiqui and Khan, 2012). The virulence properties of T4 genotype are greater than other genotypes, and that could be the reason behind the propagation of T4 genotype. It contains seven subtypes including T4A, T4B, T4C, T4D, T4E, T4F and T4-Neff. In addition, the T4 genotype secretes serine protease with different molecular weights.

Some of them produce extracellular serine proteases with approximately 33, 36, 49 and 66 kDa; and 107 kDa; however, other studies showed the presence of 27, 47, 60, 75, 100, and 110 kDa serine proteases (Khan, 2009). This variation in the molecular weight of serine proteases might correlate with the subtypes of T4 genotype. Thus, it would be very important to identify the serine proteases produced by using zymography gels. This is the first study in worldwide to report the presence of *Acanthamoeba* in wound infections.

Chapter Six

6

General discussion

Chapter 6 : General discussion

6.1 Overview

Skin is the first protective barrier of the body, however, when it is compromised, bacteria and other microorganisms may invade and colonise superficial and deep tissue (Lacey *et al.*, 2016). It has been estimated that wound infections are the most common nosocomial diseases, mostly caused by MRSA and *P. aeruginosa* (Eardley *et al.*, 2011). Several studies showed the presence of these bacteria in non-healing wounds, such as pressure sores, and diabetic foot ulcers. In addition, many other studies showed that the presence of MRSA and *P. aeruginosa* lead to the formation of resistant biofilms (Pfaller *et al.*, 1989). The pathogenicity of bacteria depends on their virulence factors including the ones responsible for binding, invasiveness and toxicity. Pathogenesis involves the beginning of the inflammation process which leads to the in part to the signs and symptoms of infection (Beceiro *et al.*, 2013). In order to investigate the reason behind episodes of wound re-infection, clinical isolates of MRSA and *Pseudomonas* isolated from wounds were used in this study, together with KB epithelial cell line and mesenchymal stem cells. Steps were taken to test if the presence of the ubiquitous *Acanthamoeba* in wounds is one of the key factors for such re-infections, as they feed on bacteria.

6.1.1 Pathogenicity of MRSA and *P. aeruginosa* in wounds

In this study, the interaction of MRSA and *Pseudomonas* with human KB epithelial cell line were first studied. Although both MRSA and *Pseudomonas* showed their ability to bind with, invade, survive and multiply inside these cells, *Pseudomonas* were more likely to interact with cells. The findings showed that the number of *Pseudomonas* bind with epithelial cells were higher compared with MRSA after 60 min incubation. The

ability of intracellular *Pseudomonas* to survive and then multiply was higher than that of MRSA. The differences in bacterial structure and their virulence factors could be the reason behind these findings.

It is well known that MRSA have some virulence factors responsible for binding to cells, such as FnBPs, protein A, elastin-binding protein and collagen-binding protein. In addition, MRSA produce different toxins and enzymes, such as Haemolysin α -toxin (Hla) and Panton-Valentine leukocidin (PVL) to invade host cells (Sinha *et al.*, 1999; Gordon and Lowy, 2008; Yang and Ji, 2014). Both Hla and PVL have multiple functions including pore-formation, utilizing iron from haemoglobin and causing cell death (Otto, 2010; Pishchany *et al.*, 2010; Lipinska *et al.*, 2011).

Pseudomonas as Gram-negative bacteria have other special molecules, such as pili and LPS, which are responsible for adherence to the surface of the host cells (Ben *et al.*, 2011). Adding to that, type III system- secretion (T3SS) is one of the major systems in *P. aeruginosa* responsible for secreting and delivering toxins, such as ExoS, ExoT, ExoY and ExoU (Schaber *et al.*, 2004; Galle *et al.*, 2012a, 2012b; Pastar *et al.*, 2013). Quorum sensing (QS) systems of *Pseudomonas*, including LasI-LasR and RhII-RhIR, are responsible for wound infection and biofilm formation (Rumbaugh *et al.*, 1999; Nakagami *et al.*, 2011). Exotoxin A is the most toxic protein compound produced by *P. aeruginosa* and is regulated by QS. It is secreted as an inactive pro-toxin which specifically interacts with the receptor present on the surface of the host cell causing a shutdown of protein synthesis resulting in cell death (Ben *et al.*, 2011).

Cytotoxic effects of MRSA and *P. aeruginosa* on KB epithelial cell line were determined by two different techniques: flow cytometry and colorimetric assays. Cytotoxic effects lead to cell death, induced by bacterial toxins and their multiplication

inside cells. In this study, live and heat-killed bacteria and BCM of MRSA and *P. aeruginosa* were used to investigate the role of the caspase-3 pathway in cell death. As expected, findings showed that the ability of live bacteria to induce cell death was higher compared with heat-killed bacteria and BCM. It may be because viable bacteria are able to multiply and produce toxins. Cell death induced by *P. aeruginosa* was greater than cell death induced by MRSA. The inactive form (heat-killed) of bacteria and BCM were able to induce cell death. However, the exact trigger for the cell death of KB epithelial cells in this study is unknown. This process was followed and apoptosis and necrosis were found following exposure to MRSA and *P. aeruginosa*. This may be due to secondary necrosis following apoptosis, or may reflect direct cytotoxic effect as well as apoptosis (Worgall *et al.*, 2002). In addition, these bacteria have the ability to decrease the expression of Keratinocyte Growth Factor 1 (KGF1), which is one of the important responsible factors needed in the wound healing process. A recent study showed that increased expression of pro-inflammatory cytokines TNF α and IL-1 β has been associated with polymicrobial biofilm formation by MRSA and *P. aeruginosa* (Pastar *et al.*, 2013). This is responsible for prolonging the inflammatory stage of the healing process.

6.1.2 Presence and role of *Acanthamoeba* in wounds

Several lines of evidence had been published, suggesting the probability of the presence of *Acanthamoeba* in wounds. First, the wide distribution of *Acanthamoeba* spp. in the environment (Lorenzo-Morales *et al.*, 2006; Chomicz *et al.*, 2010), which could lead to contaminated wounds. Secondly, skin lesions may provide the direct entry route for *Acanthamoeba* to enter the bloodstream and then interact with the BBB causing AGE (Alsam *et al.*, 2005b; Khan, 2009). Finally, the relationship between bacteria associated

with wound infections and *Acanthamoeba* (Cardas *et al.*, 2012). These lines of evidence suggest that *Acanthamoeba* could be present in wounds and therefore have a role in their infection.

In this study, the presence of *Acanthamoeba* in wounds was investigated. 140 wound swabs were collected from different sites including feet (heels and toes), knees, hips, lips, hands, breasts and thighs. Only one sample was positive for *Acanthamoeba* spp. As expected, this *Acanthamoeba* isolate was from one patient who had more than one episode of inflammation. This supports the hypothesis of this study: presence of *Acanthamoeba* in wounds might have a role in the increasing of incidence of wound reinfection. Sequencing of the highly variable DF3 region of the 18S rRNA gene of the isolate showed that this was very similar to genotype T4 subtype A. A study showed the presence of *Acanthamoeba* trophozoites and cysts in a skin nodule biopsy from a patient who had recently undergone lung transplantation (D'Auria *et al.*, 2012).

To investigate whether *Acanthamoeba* could interact with the most common bacteria in wounds, interaction assays were performed. Clinical isolates of MRSA and *Pseudomonas* were used with three different *Acanthamoeba* species, including T1, T4 and T7. Findings of this study showed that both MRSA and *Pseudomonas* were able to bind with, invade, survive and multiply inside all *Acanthamoeba* species. The nature of the relationship between bacteria and amoebae depends on bacterial virulence and amoebic virulence and also on the environmental conditions. It is interesting to mention that the number of bacteria which survived and multiplied inside the pathogenic *Acanthamoeba* T1 was very low compared with the intracellular bacteria inside *Acanthamoeba* T7. The limitation of bacterial survival inside pathogenic species compared with non-pathogenic ones may be due to proteases and toxins produced by

pathogenic *Acanthamoeba*. A study showed that, *in vitro*, clinical and non-clinical isolates belonging to genotypes, T1, T2, T3, T4 and T7 showed ecto-ATPase activities. *Acanthamoeba* binds to host cells using their mannose-binding protein and binding can be blocked using exogenous α -mannose (Alsam *et al.*, 2003). It has been demonstrated that α -mannose significantly increased ecto-ATPase activities of pathogenic *Acanthamoeba* belonging to T1, T2, T3 and T4 genotypes but not the non-pathogenic species belonging to T7 genotype (Sissons *et al.*, 2004). In addition, pathogenic *Acanthamoeba* belong to T1 and T4 genotypes were found producing extracellular proteases, such as serine and metalloproteases, which were not produced by non-pathogenic T7 genotype (Khan *et al.*, 2000; Alsam *et al.*, 2005b). Another study showed the antimicrobial activity of *A. castellanii* conditioned medium on MRSA and *P. aeruginosa* (Iqbal *et al.*, 2014). Several studies have reported the ability of bacteria to interact with amoeba, such as *Escherichia coli* (Alsam *et al.*, 2006), *Enterobacter aerogenes*, *Aeromonas hydrophila* (Yousuf *et al.*, 2013), *Pseudomonas aeruginosa* (Michel *et al.*, 1995) and MRSA (Cardas *et al.*, 2012). The relationship between bacteria and amoebae could be symbiotic or destructive of either the bacterium or the amoeba (Jeon, 1995). It has been reported that in the absence of food, pathogenic *Escherichia coli* strain K1 was able to remain viable and even multiply inside *Acanthamoeba* compared with non-pathogenic strain *E. coli* K12 which was killed. However, in the presence of variable conditions, *E. coli* K1 lysed amoebae and grew exponentially whereas *E. coli* K12 exhibited minimal growth (Alsam *et al.*, 2006). Overall, these findings suggest that *Acanthamoeba* facilitate bacterial survival and provide protection from unfavourable conditions.

6.1.3 Role of microorganisms in wound healing

Wound healing requires specific factors to interact together, including cells, growth factors, and extracellular matrix proteins. Endogenous MSC are required to accelerate wound healing by recruiting other host cells and secreting growth factors and matrix proteins. Self-renewal is the most important feature of MSC along with their ability to differentiate to other cell types (Maxson *et al.*, 2012; Ma *et al.*, 2014). However, microorganisms have the ability to delay wound healing by producing toxins and enzymes (Pastar *et al.*, 2013; Losa *et al.*, 2014).

Live, heat-killed bacteria and BCM of MRSA and *P. aeruginosa*, and *Acanthamoeba* CM were used against human skin cells and MSC to determine the effective agent of cell death. In this study, findings showed that all microorganisms induced cell death in both KB epithelial and MSC cells. As mentioned earlier, live bacteria showed a greater effect on host cells compared with heat-killed bacteria and BCM. Unfortunately, clinical isolates of MRSA and *P. aeruginosa* were not characterised as to the level of virulence factors they produce. Therefore, it is impossible to ascribe the cytotoxicity to specific virulence factors. A previous study showed that incubating inactivated (heat-killed) *P. aeruginosa* with dendritic cells for 3 h induced apoptosis (Worgall *et al.*, 2002). In this study, cell death induced by *P. aeruginosa* was less effective in KB epithelial cell line at the same conditions. In addition, BCM of both MRSA and *P. aeruginosa* were toxic on both KB epithelial and MSC cells. T3SS of *P. aeruginosa* were demonstrated to have a direct role in cell death (Losa *et al.*, 2014; Broquet and Asehnoune, 2015; Wood *et al.*, 2015). Furthermore, TSST-1 and FnBPs produced by MRSA are able to induce cell death in different cell types, such as epithelial and endothelial cells (Essmann *et al.*, 2003; Mateo *et al.*, 2007). The ability of *Acanthamoeba* trophozoites to induce

apoptosis in human corneal epithelial cells has been confirmed (Zheng *et al.*, 2004). In this study, *Acanthamoeba* CM were highly induced cell death in both KB epithelial and MSC cells. *Acanthamoeba* produce different enzymes which are able to induce apoptosis or necrosis, such as ecto-ATPases and extracellular serine proteases (Sissons *et al.*, 2004; Alsam *et al.*, 2005b; Lorenzo-Morales *et al.*, 2015). In addition, *Acanthamoeba* activates Phosphatidylinositol 3-Kinase (PI3ks) which induce brain endothelial cell death (Sissons *et al.*, 2005).

The mechanism of caspase-mediated cell death has been detected in different cell types, including epithelial cells and stem cells. Among the caspases, activation of caspase-3 plays an important role in infections as it is activated by both the intrinsic and extrinsic pathways (Hurt *et al.*, 2003; Alaoui-El-Azher *et al.*, 2006; Maxson *et al.*, 2012; Tripathi *et al.*, 2012; Soong *et al.*, 2015). Findings of this study confirmed the role of caspase-3 pathways in cell death mediated by MRSA, *P. aeruginosa* and *Acanthamoeba*.

6.1.4 Alternative efficient treatment strategy

The overuse and misuse of antibiotics result in mutations of bacteria, which lead to antibiotic resistance. Both MRSA and *P. aeruginosa* are multi-drug resistant. MRSA and *P. aeruginosa* have been directly associated with severe and chronic wound infections which result in increased morbidity and mortality rates (Gardner *et al.*, 2001; Pastar *et al.*, 2013). Treatment of multi-drug resistant bacteria is difficult, because of their increased virulence factors, such as penicillin-binding protein 2A (PBP2A) in MRSA (Peacock and Paterson, 2015). In addition to bacteria, *Acanthamoeba* cysts are very difficult to treat (Aksozek *et al.*, 2002). Therefore, there is an urgent need for novel and alternative treatment methods, such as iron chelation therapy. The antimicrobial activity of iron chelation therapy has been studied against different types of bacteria,

such as *S. aureus*, *E. coli* and *P. aeruginosa* (Hider and Kong, 2010; Qiu *et al.*, 2011). The chemical structure of iron chelators plays an important role in chelation. For examples, deferiprone is a small molecule which forms a bidentate ligand with one iron molecule, but iron chelators which are able to form a hexadentate ligand with one iron molecule have been demonstrated as high affinity and more effective on the viability of bacteria (Qiu *et al.*, 2011; Xu *et al.*, 2011; Thompson *et al.*, 2012; Zhou *et al.*, 2015).

In this study, three novel hexadentate iron chelators together with deferiprone were used to determine whether iron chelation is a promising method in the treatment of multi-drug resistant microorganisms. All novel iron chelators showed antimicrobial activity against both extracellular and intracellular bacteria inside host cells and *Acanthamoeba*. Iron chelators investigated in previous studies vary in their core and novel compounds (Qiu *et al.*, 2011; Xu *et al.*, 2011; Thompson *et al.*, 2012; Zhou *et al.*, 2015). In one study, the MIC of deferiprone against *S. aureus* and *P. aeruginosa* was over 500 µg/mL (Thompson *et al.*, 2012) which is in agreement with our findings. The most effective iron chelator against MRSA and *P. aeruginosa* in this study was 13l, with MIC of >500 and 400 µg/mL, respectively. By comparison the MICs of another novel hexadentate iron chelator CP251 against MRSA and *P. aeruginosa* were 500 and 100 µg/mL, respectively (Qiu *et al.*, 2011). As well as that, all iron chelators used in this study showed very low toxicity on host cells, which was determined by LDH assays.

This was the first report showing the use of iron chelation against *Acanthamoeba*. However, the use of iron chelators against *Naegleria fowleri* and *Entamoeba histolytica* has been investigated (Newsome and Wilhelm, 1983; Espinosa *et al.*, 2009). Our findings showed that the MIC of 13a, 13l and 13i on both *Acanthamoeba* T4 and T7 were 100, 300 and 400 µg/mL, respectively, after 24 h incubation. The MIC of

desferrioxamine B against *Naegleria fowleri* was found to be 1000 µg/mL after 96 h (Newsome and Wilhelm, 1983).

6.2 Limitations of the study

The use of two types of cell lines, three *Acanthamoeba* species, three MRSA and three *Pseudomonas*, was a unique feature of this study. Several techniques were used in this project including interaction assays, antimicrobial activity (MBC and MIC), LDH cytotoxicity colorimetric assays, zymographic assays, flow cytometry, PCR and bioinformatics. On the other hand, there were limitations of this study.

One of the limitations of this study was that the KB epithelial cell line was derived from a cancerous tissue and does not represent the skin tissue of a healthy individual. This cell line may not express the same receptors as normal skin epithelial cells, therefore the interaction of bacteria with the KB epithelial cell line may not be representative of the interaction which would take place in a healthy individual. Another type of cell which could be considered for use would be the normal human keratinocyte cells which are derived from a healthy individual foreskin. In addition, mMSC cell which was used in this study was isolated from murine bone marrow, which could be different from human MSC.

Clinical isolates of MRSA and *Pseudomonas* were used in this study. The virulence factors of individual strains have not been characterised. To study individual virulence factors, inhibitors or mutant strains lacking one or more virulence factors could be used.

6.3 Recommendations for future work

The findings presented in this thesis highlighted some aspects of *Acanthamoeba* role in wound infections, along with the pathogenesis of MRSA and *P. aeruginosa*, and the promise of using iron chelation as an alternative therapeutic method. There are, however, needs for further investigations to give a better picture for *Acanthamoeba*'s role in wounds.

1. Further investigations of the virulence factors most responsible for cytotoxicity could be worth studying. To assess that, a few cell types derived from healthy human skin tissue are required, such as keratinocytes, fibroblasts and endothelial, or a skin tissue model. In addition, different bacterial strains lacking one or more of the virulence factors could be used on the above-mentioned cells. Many techniques would be helpful to assess that: at proteins level, such as flow cytometry, fluorescence microscopy, and western blot using antibodies; and at the gene level, such as PCR, sequencing and bioinformatics.

2. Another matter would be using human MSC to study the effect of different virulence factors in cell death. As well as that, understanding the pathways of cell death will shed some more light in this field.

3. It is well known that biopsies from infected wounds are better samples compared with swabs for isolation of any microorganisms (Central Disease Control and Prevention, 2014). Therefore, collecting biopsies to detect the presence of *Acanthamoeba* in infected wounds could be a future study.

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Appendices

Appendix 1: List of reagents and chemicals

7.1 List of reagents and chemicals

7.1.1 Reagents and media for bacterial, amoebal and cell culture

Minimum Essential Medium Eagle (EMEM)	(Sigma M0644)
Dulbecco's Modified Eagle's Medium (DMEM)	(Gibco™ 61965026)
MEM Non-Essential Amino Acid Solution (100×)	(Sigma 7145)
Fetal Bovine Serum	(Fisher Scientific 10117272)
Phosphate buffered saline (PBS)	(Sigma P4417)
Nutrient agar	(Sigma N9405)
Bacteriological agar	(Sigma A5306)
CLED Agar	(Sigma 55420)
Ringers solution	(Sigma 96724)
D-(+)-Glucose	(Sigma G8270)
Peptone Water	(Sigma 70179)
Yeast Extract	(Sigma 92144)
Trypsin-EDTA solution	(Sigma T4174)
Sodium chloride (NaCl)	(Sigma S7653)
Tryptone	(Sigma T7293)
Accutase® solution	(Sigma A6964)
Sodium phosphate dibasic – potassium phosphate monobasic solution	(Sigma 71653)
Magnesium sulfate heptahydrate	(Sigma M1880)
Calcium chloride dihydrate	(Sigma 223506)

7.1.2 Antibiotics and antimicrobial agents

Vancomycin	(Sigma V2002)
Ciprofloxacin	(Sigma 17850)
Deferiprone	(Sigma 379409)

7.1.3 Reagent for Zymography

Triethylamine hydrochloride (Tris)	(Sigma T8521)
Bis/ acrylamide solution for electrophoresis	(Sigma A3574)
Sodium dodecyl sulfate (SDS)	(Sigma L3771)

Glycine	(Sigma G8898)
Glycerol	(Sigma G5516)
2-mercaptoethanol	(Sigma M6250)
Bromophenol blue	(Sigma B0126)
Coomassie brilliant blue	(Sigma B0149)
TEMED	(Sigma T9281)
Ammonium persulphate	(Sigma A3678)
Acetic acid	(Fisher Scientific 10375020)
Methanol	(Fisher Scientific 10093880)
Triton X-100	(Sigma X-100)
Calcium chloride (CaCl₂)	(Sigma C1016)
Gelatin	(Sigma 48723)
7.1.4 Kits	
QIAamp DNA Micro Kit	(QIAGEN 56304)
QIAquick Gel Extraction Kit	(QIAGEN 28704)
Cytotoxicity Detection Kit (LDH)	(ROCHE 11644793001)
7.1.5 Inhibitors	
Phenylmethanesulfonyl fluoride (PMSF)	(Sigma P7626)
General caspase inhibitor (Z-VAD-FMK)	(BD 550377)
7.1.6 Reagents for flow cytometry	
Annexin V FITC	(BioLegend 640906)
Annexin V Binding Buffer	(BioLegend 422201)
Cell staining buffer	(BioLegend 420201)
Zombie NIR	(BioLegend 423106)
7.1.7 Reagents for PCR and gel electrophoresis	
Agarose	(Fisher Scientific 10776644)
GeneRuler 1 kb DNA Ladder	(Thermo Scientific™ SM0311)
dNTP Mix	(Thermo Scientific™ R0241)
Taq DNA Polymerase	(Thermo Scientific™ EP0401)
SafeView Nucleic Acid Stain	(NBS-Biologicals NBS-SV1)
7.1.8 General reagents	
Ethylenediaminetetraacetic acid (EDTA) (10×)	(Sigma E9884)

Acetone	(Fisher Scientific 10045800)
Hematoxylin	(Sigma H9627)
CellLytic™ M	(Sigma C2978)
Virkon	(Fisher Scientific 12358667)
Ethanol	(Fisher Scientific BP2818)
Dimethyl Sulfoxide (DMSO)	(Sigma D2650)

Appendix 2: List and preparation of buffers and cultured medium

7.2 List and preparation of buffers and cultured medium

7.2.1 Saline

Page's Amoeba Saline:

Solution1:

- Sodium phosphate dibasic (Na_2HPO_4) 0.142 g
- Potassium phosphate monobasic (KH_2PO_4) 0.136 g
- DI Water 500.0 mL

Autoclave at 121°C for 15 min

Solution2:

- Magnesium Sulfate Heptahydrate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) 4.0 mg
- Calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$) 4.0 mg
- Sodium chloride (NaCl) 0.120 g
- DI Water 500.0 mL

Autoclave at 121°C for 15 min

Combine solutions 1 and 2 when cooled to room temperature.

Ringers solution 1/4 strength tablets: One tablet is sufficient for 500mL contained:

- calcium chloride, 0.12 g/l
- potassium chloride, 0.105 g/l
- sodium bicarbonate, 0.05 g/l
- sodium chloride, 2.25 g/l

Phosphate buffered saline (PBS) tablets: One tablet dissolved in 200 mL of deionized water giving 0.01 M phosphate buffer contained:

- potassium chloride, 0.0027 M
- sodium chloride, 0.137 M

Autoclave at 121°C for 15 min. pH 7.4, at 25 °C

7.2.2 Human cell, amoebal, and bacterial culture medium

EMEM for KB epithelial cell line (v/v): EMEM 89 %, NEAA 1 %, and FBS 10 %.

DMEM for MSC cell (v/v): DMEM 80 %, and FBS 10 %.

PYG (w/v): proteose peptone 0.75 %, yeast extract 0.75 %, and glucose 1.5 % distilled water prior to autoclaving 121°C for 15 min.

Luria Bertani broth (w/v): 10g tryptone, 5g yeast extract and 10g NaCl in water to a final volume of 1000 mL, adjusting the pH to 7.5 before autoclaving at 121°C for 15 min.

CLED (w/v): 36.15 g of CLED agar dissolved up to 1000 mL distilled water prior to autoclaving 121°C for 15 min.

Nutrient agar (w/v): 23 g of nutrient agar dissolved up to 1000 mL distilled water prior to autoclaving 121°C for 15 min.

Non-nutrient agar (w/v): 30 g bacteriological agar dissolved in to 1000 mL Ringers solution, or in Page amoeba saline prior to autoclaving 121°C for 15 min.

Trypsin 1x (v/v): 10 mL up to 100 mL PBS.

10 % SDS (w/v): 10 g dissolved up to 100 mL distilled water.

7.2.3 Antibiotics and antimicrobial iron chelator

Vancomycin (w/v): stock solution: 1 mg/mL dissolved in distilled water

Ciprofloxacin (w/v): stock solution: 1 mg/mL dissolved in distilled water with 0.05mM HCl

Deferiprone (w/v): stock solution: 10 mg/mL dissolved in distilled water.

13a (w/v): stock solution: 10 mg/mL dissolved in distilled water.

13l (w/v): stock solution: 10 mg/mL dissolved in distilled water.

13i (w/v): stock solution: 10 mg/mL dissolved in distilled water.

7.2.4 Buffers for electrophoresis:

SDS-PAGE (Zymography):

Some of SDS-PAGE reagents and buffers were prepared in the laboratory before using, and others were purchased from sigma. pH for all reagents and buffers were adjusted by using either HCl or NaCl. SDS-PAGE reagents and buffers were prepared as follows:

- **30 % (v/v) Bis/acrylamide solution** for electrophoresis.
- **Temed** solution.
- **Tris:** were prepared in two concentrations:
 - a) 1.5M Tris (pH 8.8): 18.1g of Tris were dissolved in 100mL of distilled water.
 - b) 1M Tris (pH 6.8): 12.05g of Tris were dissolved in 100mL of distilled water.
- **10 % SDS (w/v):** 10g of SDS were dissolved in 100mL of distilled water.
- **10 % Ammonium persulphate:** 0.1g of ammonium persulphate (w/v) were dissolved in 1mL of distilled water.
- **Running buffer** (pH 8.3): 15g of Tris (v/v), 72g of glycine (v/v) and 5g of SDS (v/v)

were dissolved in 1000mL of distilled water. Running buffer was diluted with distilled water 1:1.

- **Sample buffer:** 4mL of distilled water, 1mL of 0.5M Tris (v/v), 0.8mL of glycerol (v/v), 1.6mL of 10 % SDS (v/v), and 0.05 % bromophenol blue (w/v in distilled water) were mixed together.
- **Coomassie brilliant blue stain:** Coomassie brilliant blue (w/v), methanol (v/v), acetic acid (v/v) and distilled water (v/v) were mixed as (1:8:2:10).
- **De-stain:** methanol (v/v), acetic acid (v/v) and distilled water (v/v) were mixed as (4:1:5).
- **Tris buffer** used to prepare triton and developing buffer: 50mM Tris (6.057g/l), adjust pH to 7.5.
 - a) **Triton (v/v):** 2 % Triton X-100 in Tris buffer.
 - b) **Developing buffer (w/v):** 10 mM CaCl_2 1.4 g/l in Tris buffer.

Agarose gel:

0.5 M EDTA: use stirring hotplate to 50-60 °C for 30-60 mins, pH8 once redissolved.

10 % SDS-PAGE running gels		5 % Stacking gel	
Distilled water	4.2 mL	Distilled water	4.3 mL
30 % Bis/acrylamide solution	3.3 mL	30 % Bis/acrylamide solution	0.8 mL
1.5M Tris (pH 8.8)	2 mL	1.0M Tris (pH 6.8)	0.75 mL
10 % SDS	100 μL	10 % SDS	60 μL
10 % Ammonium persulphate	400 μL	10 % Ammonium persulphate	250 μL
TEMED solution	6 μL	TEMED solution	3 μL
4 % Gelatin	400 μL		

50X TAE buffer: Stock solution:

- Tris base (w/v) 242g
- Glacial Acetic Acid (v/v) 57.1 mL
- 0.5M EDTA pH8 (v/v) 100 mL

Dissolve Tris in ~600 mL ddH₂O, then EDTA and Acetic Acid were added, up to 1000 mL with H₂O.

1X TAE buffer: 20 mL of 50X TAE buffer up to 1000 mL of H₂O.

1 % Agarose gel: 40 g agarose dissolved in 400 mL 1X TAE buffer at 50 °C then 1mL of

safeview were added and mixed prior to poured in electrophoresis system tray.

7.2.5 Primers preparation

1. Spin the tubes at top speed for 10 min to ensure that there are no lyophilized primers stuck to the cap.
2. To make a typical 100 mM (100X) stock concentration of primers, dissolve the primers in a volume of sterile distilled water that is 10X the amount of nmoles in the tube, using μL of water. This value is printed on the side of the tube. For example, if your tube contains 53.4 nmoles of primer, then you would dissolve using 534 μL of water. This will now be at a 100 μM concentration.

i.e. 53.4 nmoles of primer dissolve in 534 μL of water =

$$(53.4 \text{ nmoles/l}) \times (534 \times 10^{-6} \text{ l}) = 0.0001 \text{ mole/l} = 0.0001 \text{ M} = 0.1 \text{ mM} = 100 \mu\text{M}$$

3. To prepare primers for use:

Dilute this stock 1:10, to give a concentration of 10 μM .

4. Store all sets of primers at -20°C .
5. Typically use 0.1-1.0 μM final concentration in a PCR mix. i.e. if your reaction volume is 25 mL, then using 2.5 μL for each separate primer, will give you a final concentration of 1 μM .

7.2.6 Disinfections used in the laboratory:

1 % virkon (w/v): 1 mg in 100 mL PBS

70 % ethanol (v/v): 70 mL in 100 mL H_2O

Appendix 3: List of cell lines and microorganisms; and storage and thawing methods

7.3 List of cell lines and microorganisms; and storage and thawing methods

7.3.1 List of Cell lines

KB epithelial (human epidermal carcinoma) cell line was provided from University College London.

mMSCs (murine mesenchymal stem cells) primary cells was provided by Dr Ralf Zwacka from University of Essex.

7.3.2 List of *Acanthamoeba* strains

<i>Acanthamoeba castellanii</i>	T1	(ATCC 50494)
<i>Acanthamoeba castellanii</i>	T4	(ATCC 30234)
<i>Acanthamoeba astronyxis</i>	T7	(ATCC 30317)

7.3.3 Storage method for cells and *Acanthamoeba*

Cells and *Acanthamoeba* were stored as axenic cultures at -80°C and liquid nitrogen. Briefly, growing cells or amoebae were re-suspended at a density of $1-5 \times 10^6$ cells/mL in the freezing medium [90 % FBS, and 10 % DMSO (v/v) for human cells; 90 % PYG, and 10 % DMSO (v/v) for *Acanthamoeba*] and placed into a cryotube. The freezing process were done gradually in ice, then -20 °C for 60 min followed by their storage at -80°C freezer. Some of cryotubes were transferred after overnight incubation in -80 °C to store in liquid nitrogen.

7.3.4 Thawing method for cells and *Acanthamoeba*

Thawing and recovery of stored cells and *Acanthamoeba* from both liquid nitrogen and -80°C freezer was done quickly. The cryotubes were placed immediately in the warmer incubator 37°C for 3 min after taken out from liquid nitrogen or -80 °C freezer. Cells or *Acanthamoeba* were transferred into a 15 mL tube with warmed medium (EMEM for KB epithelial cell line, DMEM for MSC, and PYG for *Acanthamoeba*) and centrifuged at 2000 x g for 5 min. Next, supernatants were discarded, then pellets were resuspended in owned medium (EMEM for KB epithelial cell line, DMEM for MSC, and PYG for *Acanthamoeba*) and transferred into a T25 tissue flasks. Flasks were incubated at 37 °C, 5 % CO₂ and 95 % humidity for KB and MSC cells or 30 °C air incubator for *Acanthamoeba* until the cells reached an enough number to transfer.

7.3.5 Bacterial strains

Clinical isolates of MRSA and *Pseudomonas* were generously provided by Dr Tony Elston from Colchester General Hospital.

E. coli K12 (ATCC 33876) was provided by Dr Terry McGenity from University of Essex.

7.3.6 Storage of bacterial cultures

By using a disposal sterile loop, about 20-30 isolated colonies of bacteria were picked up from the agar plate and inoculated in a 10 mL of bacterial freezing medium [70 % Luria-Bertani broth and 30 % glycerol (v/v)]. Then, the mixture of freezing medium with bacteria were divided into cryotubes final volume of each tube was 0.5 mL and stored in a -20 °C freezer.

7.3.7 Thawing and re-culturing bacteria

To re-culture bacteria, a disposable sterile loop was inserted into the frozen cryotube and then streaked on fresh agar plate and incubated at 37 °C air incubator overnight. After the incubation, the plate was carefully examined if the colonies have similar morphologies such as textures, colours and border shapes and stored at 4 °C for up to a month.

Appendix 4: Approvals documents

7.4 Approvals documents

7.4.1 Ethical approval letter



NRES Committee London - Chelsea
Research Ethics Committee (REC) Bristol Centre
Level 3, Block B
Whitefriars
Lewins Mead
Bristol
BS1 2NT

Telephone: 0117 342 1380

20 August 2014

Mr Osamah Al Rugai
3 Saran Court
Wivenhoe
Colchester
CO7 9RS

Dear Mr Al Rugai

Study title:	Role of Acanthamoeba in wound infections
REC reference:	14/LO/1538
Protocol number:	N/A
IRAS project ID:	138584

The Proportionate Review Sub-committee of the NRES Committee London - Chelsea reviewed the above application on 18 August 2014.

We plan to publish your research summary wording for the above study on the NRES website, together with your contact details, unless you expressly withhold permission to do so. Publication will be no earlier than three months from the date of this favourable opinion letter. Should you wish to provide a substitute contact point, require further information, or wish to make a request to postpone publication, please contact the REC Manager Miss Gemma Oakes, nrescommittee.london-chelsea@nhs.net.

Ethical opinion

On behalf of the Committee, the sub-committee gave a favourable ethical opinion of the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

The favourable opinion is subject to the following conditions being met prior to the start of the study.

You confirmed the School of Biological Science in University of Essex followed the Human Tissue Authority Code of Practice in disposing of any human materials and methods were available if required.

Approved documents

The documents reviewed and approved were:

Document	Version	Date
Evidence of Sponsor insurance or indemnity (non NHS Sponsors only) [(Ref: 5067424)]		06 August 2014
IRAS Checklist XML [Checklist_11082014]		11 August 2014
Letter from sponsor [(University of Essex)]		29 July 2014
REC Application Form		07 August 2014
Referee's report or other scientific critique report		04 August 2014
Research protocol or project proposal	1.0	20 May 2014
Summary CV for Chief Investigator (CI) [Osamah Al rugaie]		
Summary CV for supervisor (student research) [Selwa Alsam]		
Summary, synopsis or diagram (flowchart) of protocol in non -technical language	1.0	04 August 2014

Membership of the Proportionate Review Sub-Committee

The members of the Sub-Committee who took part in the review are listed on the attached sheet.

There were no Declarations of Interest.

Statement of compliance

The Committee is constituted in accordance with the Governance Arrangements for Research Ethics Committees and complies fully with the Standard Operating Procedures for Research Ethics Committees in the UK.

After ethical review

Reporting requirements

The attached document "After ethical review – guidance for researchers" gives detailed guidance on reporting requirements for studies with a favourable opinion, including:

- Notifying substantial amendments
- Adding new sites and investigators
- Notification of serious breaches of the protocol
- Progress and safety reports
- Notifying the end of the study

The HRA website also provides guidance on these topics, which is updated in the light of changes in reporting requirements or procedures.

User Feedback

The Health Research Authority is continually striving to provide a high quality service to all applicants and sponsors. You are invited to give your view of the service you have received and the application procedure. If you wish to make your views known please use the feedback form available on the HRA website:

<http://www.hra.nhs.uk/about-the-hra/governance/quality-assurance/>

HRA Training

We are pleased to welcome researchers and R&D staff at our training days – see details at <http://www.hra.nhs.uk/hra-training/>

With the Committee's best wishes for the success of this project.

14/LO/1538

Please quote this number on all correspondence

Yours sincerely



Dr Michael Schachter
pp Alternate Vice Chair

Email: nrescommittee.london-chelsea@nhs.net

Enclosures: *List of names and professions of members who took part in the review
"After ethical review – guidance for researchers" [SL-AR2]*

Copy to: *Mr Ayres Caldeira, ayres.caldeira@colchesterhospital.nhs.uk*

NRES Committee London - Chelsea

Attendance at PRS Sub-Committee of the REC meeting on 18 August 2014

Committee Members:

<i>Name</i>	<i>Profession</i>	<i>Present</i>	<i>Notes</i>
Mr Roger A'hern	Senior Statistician, Clinical trials and statistics unit	Yes	
Mr Philip Kimberley	Clinical Governance Information Manager	Yes	
Dr Michael Schachter (Chair)	Clinical Pharmacologist	Yes	

Also in attendance:

<i>Name</i>	<i>Position (or reason for attending)</i>
Miss Gemma Oakes	REC Manager

7.4.2 Research and development approval

Colchester Hospital University 
NHS Foundation Trust

Colchester General Hospital
Microbiology Department
Turner Rd
Colchester
CO4 5JL

Tel: 01206 747374

Dr Selwa Alsam
Biological Sciences
University of Essex

Date: Wednesday, 28 January 2015

Your Ref:

Our Ref:

Dear Selwa,

Re; Osamah Al Rugaie

We would like to give access to Osamah to the laboratory so that he may conduct part of his research project. We understand that he will be accompanied by yourself and the impact on our staff time will be minimal.

Kind regards

Yours sincerely



R A Elston
Consultant Microbiologist



Chief Executive (Interim): Kim Hodgson
Chair: Dr. Sally Irvine

Publications and Conferences

Publications and Conferences

Publications:

2016

Junpei Li; Eniola D. Olaleye; Xiaole Kong; Tao Zhou; Yongmin Ma; Jagoda Jurach; **Osamah Al Rugaie**; Robert C. Hider; Guoqing Zhang; Selwa Alsam; and Vincenzo Abbate

“Macromolecular iron-chelators via RAFT-polymerization for the inhibition of methicillin-resistant *Staphylococcus aureus* growth”. *Polymer* **87**, pp. 64-72.

2015

Ying-Jun Zhou; Mu-Song Liu; **Osamah Al Rugaie**; Xiao-Le Kong; Selwa Alsam; Sinan Battah; Yuan-Yuan Xie; Robert C. Hider; and Tao Zhou

“Hexadentate 3-hydroxypyridin-4-ones with high iron(III) affinity: Design, synthesis and inhibition on methicillin resistant *Staphylococcus aureus* and *Pseudomonas* strains”. *European Journal of Medicinal Chemistry* **94**, pp. 8-21.

Conferences (Posters):

2016

Osamah Al Rugaie; Tony Elston; and Selwa Alsam

Genotyping of potentially pathogenic *Acanthamoeba* strain isolated from an infected wound patient in Colchester. Microbiology Society Annual Conference 2016, Liverpool.

2015

Osamah Al Rugaie; Mu-Song Liu; Xiao-Le Kong; Sinan Battah; Yuan-Yuan Xie; Robert C. Hider; Tao Zhou; and Selwa Alsam

The effect of hexdentate hydroxypyridinone chelators as antimicrobial agents against MRSA and *P. aeruginosa* - 9th Saudi Student Conference in UK (SSCUK) - Imperial College – London 2015